The role of Apc in cortical development

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This thesis is dedicated to my family

Disclaimer

I (Uladzislau Ivaniutsin) composed this thesis and performed all of the experiments presented herein unless otherwise clearly indicated in the text. No part of this work has been, or is being submitted for other degree o professional qualification.

Signed:

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Abbreviations

A–P – anterior-posterior
AEP – anterior entopeduncular area
ANR – anterior neural ridge
Apc – Adenomatous Polyposis coli
AVE – anterior visceral endoderm
bHLH – basic helix–loop-helix
CGE – caudal ganglionic eminence
CNS – central nervous system
CNS – central nervous system
DP – dorsal pallium
DTel – dorsal telencephalons
D–V – dorso-ventral
FACS – Fluorescence Activated Cell Sorting
FAP – Familial Adenomatous Polyposis
HRP – horse radish peroxidase
IddU – iododeoxyuridine
IZ – intermediate zone
LGE – lateral ganglionic eminence
LP – lateral pallium
MGE – medial ganglionic eminence
MP – medial pallium
MTOC – microtubule organization centre
NES – nuclear export signal
NGF – nerve growth factor
NSC – neural stem cell
OLP – oligodendrocyte precursor
PCNA – Proliferating cell nuclear antigen
PCP – planar cell polarity
PCR – Polymerase Chain Reaction
PSB – pallial–subpallial boundary
sFRP – secreted Frizzled-related protein
SVZ – subventricular zone
Tc – cell cycle length
Tcf/Lef – T-cell factor/Lymphoid enhancer factor
Ts – S-phase length
VP – ventral pallium
VZ – ventricular zone
Zli – zona limitans intrathalamica

Abstract

Cerebral cortex development is a complicated process which is not completely understood. There are a lot of signaling interactions during morphogenesis of the cortex. One of the signaling pathways is Wnt. Adenomatous polyposis coli (Apc) is a key regulator of the canonical Wnt pathway. Also Apc is important to transducer signals from the planar cell polarity (PCP) Wnt pathway. This thesis is devoted to the role of Apc protein in the development of the mouse dorsal telencephalon. $Apc^{-/-}$ embryos die at gastrulation, therefore $Emx1^{Cre}$ conditional knock-out approach was used to overcome this problem. Cre expression driven by Emx1 promoter allows knock-out Apc in the cortex. Current work presents description and possible explanations of Apc functions in the developing cerebral cortex.

Conditional knock-out of Apc in the cortex using $Emx1^{Cre}$ leads to severe developmental defects in this region. This shows that Apc is required for normal development of the cerebral cortex. The earliest found defect is nuclear translocation of beta-catenin which is demonstrating that Apc is important to regulate translocation of beta-catenin to the nucleus. This finding supports results of other researchers. This abnormality was found at embryonic day 10.5 (E10.5), which shows that Apc has a controlling function from the beginning of cortical development. Later in development nuclear beta-catenin accumulation becomes more pronounced. Experiments with BatGal reporter mice show that stabilized beta-catenin is able to stimulate the canonical Wnt pathway. Wnt target genes (C-myc, Cyclin D1) are activated also. However Axin 2 expression is highly up-regulated which reflects negative feedback to the canonical Wnt

signaling activation. Polarity of cells is lost from E12.5, which suggests that the cytoskeletal functions of Apc are affected by Apc deletion. Adhesion defect and defect in neuronal processes elongation provide additional evidence of the cytoskeleton disregulation. Therefore, the deletion of Apc leads to over-activation of the canonical Wnt pathway and disregulation of cytoskeleton functioning.

Identity of the dorsal telencephalon is unclear in conditional Apc mutant embryos. Expression of Foxg1 shows that the mutant dorsal telencephalon loses telencephalic identity from E12.5. There are signs that the mutant dorsal telencephalon expresses markers (Pax3, Wnt1) of more caudal regions of the brain. However markers normally expressed in the cerebral cortex (Pax6, Tbr1) are still present. Pax6 is down-regulated in the mutant but there are no signs that the pallial subpallial boundary is compromised. Apical progenitor population is decreased. Decreased Tbr2 expression shows that intermediate progenitor pool is reduced also. Medial regions of the mutant dorsal telencephalon are more affected than lateral possibly due to a gradient in Emx1 expression in the cortex. My data show that Apc is important for proper patterning of the cortex probably mostly by antagonizing the canonical Wnt pathway. However a precise mechanism is yet to be elucidated.

Cell-cycle investigation revealed that in the absence of Apc, S-phase and cell cycle length remains more or less similar to the control. However the proliferative pool is decreased and most cells of the mutant are blocked in G1 phase. This defect progresses with the age. Aneuploidy was not detected in the mutant cells. The G1 blockade is related to p21 up-regulation. Also apoptosis is increased in the mutant but level of p53 is not changed. My data suggests that apoptosis and p21 expression is

stimulated by Wt1 expression, which reflects tumour suppression. Thus, Apc deletion leads to defects in maintenance of the size of progenitor pool and regulation of apoptosis.

In conclusion, the conditional Apc deletion has a pleiotropic effect on cortical development. Developmental defects are disregulation of the Wnt signaling, lost of cell polarity, adhesion defects, identity defects, and apoptosis disregulation. Assignment of these defects to particular Apc functions is not completely clear yet.

1. Genetic control of forebrain development

1.1 Introduction

The central nervous system originates from the neural plate, which develops from the ectoderm (neuroectoderm). However the other germ layers, the mesoderm, and the endoderm have critical roles in development of the central nervous system. For example, mesoderm induces formation of the neural tube, and endoderm (anterior visceral endoderm particularly) is required for head induction and maintenance (Rallu et al, 2002). The neural plate folds and when the edges of the plate merge, the neural tube forms. Later on in development brain vesicles are formed at the rostral part of the neural tube. There are, from posterior to anterior, the hindbrain vesicle, the midbrain vesicle, and the forebrain vesicle which eventually produce their respective brain parts. The forebrain, or prosencephalon, can be divided in several parts: diencephalon (future thalamus), telencephalon (develops into cerebral cortex, basal ganglia, and olfactory bulb), hypothalamus and retina.

Below is described what is known about the development of the central nervous system from early embryogenesis to the generation of the cerebral cortex, the structure on which this thesis is concentrated. Also functions of Apc (Adenomatous polyposis coli) are described in detail as the current work is devoted to its roles in the development of the dorsal telencephalon.

Data in this chapter are based mostly on results from mouse models because the mouse model is used in my thesis. Central nervous system development is a conserved

process and, therefore, results obtained in one species can be extrapolated to others (reviewed by Riechert , 2002). Therefore I also used data from other model organisms, as different organisms are suitable for different experiments.

1.2 Early stages of central nervous system development

The generation of the central nervous system depends on the formation of multiple neuronal cell types arranged in appropriate numbers and precise positions.

A broad expression of transcription factors Otx2 and Sox2 (markers of neuronal tissue) in the epiblast (the outer layer of a blastula, which gives rise to the ectoderm after gastrulation) at E6.5 suggests a broad neural induction (Ang et al., 1996; Avilion et al., 2003).It was shown that mutations of Bmp (bone morphogenic protein) receptors or Nodal led to a conversion of the epiblast to neural tissue (Di-gregorio et al., 2007; Camus et al., 2006). Therefore antagonism of Bmp and Nodal signaling is required to localize expression of Otx2 and Sox2 genes to the presumptive neural tissue region and to allow the formation of other tissues of the embryo

Formation of the nervous system begins with gastrulation when the early primitive streak (a structure that forms in the posterior region of the embryo and is the first visible sign of gastrulation) forms with a gastrula organizer localized (rostrally) to it (Kinder et al 2001). Extra-embryonic tissue, which is located posterior to the streak, expresses Bmps, while the streak, future mesendoderm (embryonic tissue that gives rise both to mesoderm and endoderm.), is a source of Wnts, Fgfs, and Nodal (Liu et al., 1999; Streit and Stern, 1999; Brennan et al., 2001). To prevent complete loss of anterior

neural tissue the gastrula organizer (becomes the node at later stages) antagonizes Bmp signaling by expressing Chordin and Noggin, whose productions are required for proper forebrain development (Bachiller et al., 2000). Chordin and Noggin are secreted Bmp antagonists expressed in the mouse from the gastrula organizer, but not in the AVE (anterior visceral endoderm). Mouse mutants that are double-homozygous for *Chordin* and *Noggin* mutations display severe defects in the development of the prosencephalon. This leads to the conclusion that Bmp antagonists in the node and its derivatives are required for proper forebrain development (Bachiller et al, 2000).

The mouse AVE, according to Kimura et al (2000), directs movement of some of the pre-forebrain cells rostrally, away from the caudalizing influence of the node. Thus, the specified anterior neural cells move toward the anterior visceral endoderm localized at the anterior (rostral) end of the embryo which is a source of Cerberus, a Wnt and Bmp signaling antagonist (Belo et al., 1997; Bouwmeester et al., 1996), thus preventing posteriorizing of neural tissue. It is interesting that null-mutation of *Cerberus* does not cause obvious defects in head formation (Shawlot et al., 2000). This suggests that establishment of neuronal identity relies on multiple redundant factors. Another Wnt antagonist Dkk1 is expressed by anterior visceral endoderm (Mukhopadhyay et al., 2001).In contrast to *Cerberus* mutation, *Dkk1* null mutant embryos develop truncation of forebrain and a portion of midbrain with head development defects but structures more caudal than the external ear show normal anterior-posterior body axis by gross morphology (Mukhopadhyay et al., 2001). This suggests that Dkk1 is an indispensable factor for brain and head development.

Interaction of signaling centers during development produces gradients of signaling molecules thus determining areas of prospective forebrain, midbrain, hindbrain, and spinal cord around E7.5 (Tam 1989).

1.3 Formation of the forebrain

Formation of the forebrain starts with the formation of an anterior low (rostral) to posterior high (caudal) gradient of Wnt (Fig 1.1). According to one model, neural induction in the presence of caudalizing factors will lead to the formation of neural tissue with caudal character, while neural induction in regions of the embryo lacking caudalizing factors will lead to induction of neural tissue with anterior character (Wilson and Houart, 2004). Therefore it is possible that anterior neural tissue must be protected from the influence of caudalizing signals. There are two possible ways for rostral neural tissue to retain anterior character. One possibility is localized caudal expression of caudalizing factors. The second is localized rostral expression of antagonists of caudalizing factors. However the early patterning of both anterior and posterior neural tissue is mediated through signals which are emitted from the primitive node or the organizer which is located in the upper (dorsal) blastopore lip (Xenopus studies by Constance et al, 2004). The organizer is a piece of embryonic tissue that creates an organization field of certain (axial) orientation and extent, in the undifferentiated material in which it is normally located or to which it is transplanted (Spemann, 1931). In addition to the organizer, anterior visceral endoderm (AVE, extra-embryonic tissue that is never a part of the node, or the organizer) acts to induce and maintain anterior neural character, counteracting posteriorizing signals (Rodriguez et al, 2005). Mutations

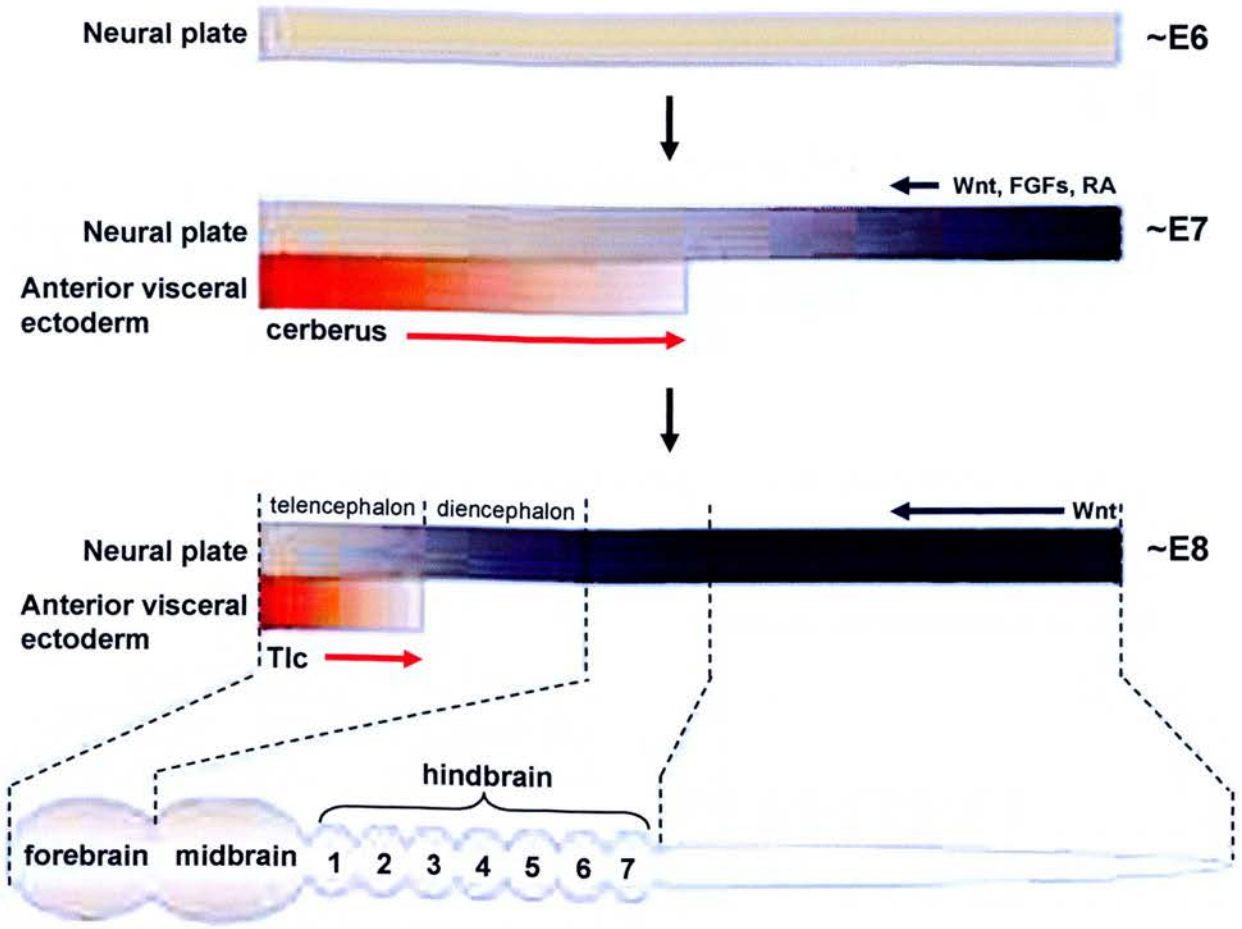


Fig 1.1. Stages of development from formation of the neural plate to subdivision of the anterior neural domain. FGF – fibroblast growth factor, RA –retinoid acid, Tlc – secreted Frizzled related protein (Wnt antagonist), E –embryonic day (modified from Rallu et al, 2002).

in genes expressed by anterior neural endoderm (*Hesx1*, a paired-like homeobox-containing gene, *Six3,* a homeodomain transcription factor, and *Otx2*, a homeodomain-containing transcription factor related to Drosophila *Otd*) (Beddington and Robertson, 1999), lead to failure of development of anterior neural structures, including forebrain. It was established that *Otx2*$^{-/-}$ embryos did not express *Dkk1* (Zakin et al., 2000) and that in *Dkk1* null embryos expression of *Otx2* is unaffected whereas *Hesx1* expression is absent in the anterior neural ectoderm (Mukhopadhyay et al., 2001); null mutation of *Hesx1* reduces *Six3* expression on early somite stages (Martinez-Barbera et al., 2000). Therefore it is possible to suggest a signaling hierarchy in the developing brain: Otx2 –> Dkk1–> Hesx1 –>Six3.

1.4 Parsing of the prosencephalon

. One of the members of the family of homeodomain transcription factors is *Six3*, which is expressed in future telencephalic and eye field regions and is crucial for formation of these structures (Lagutin et al, 2003). Increased Wnt activity or ablation of the ANR suppresses *Six3* expression; and vice versa, *Six3* activity represses transcription of *Wnt* genes (Braun et al, 2003).

Among the potential effectors of Wnt signaling is the Iroquois (Irx, Iro) family of homeodomain proteins (Itoh et al, 2002). *Iro* genes are expressed in the prospective caudal diencephalon and midbrain and promote posterior fates within prospective forebrain and midbrain forming areas. Itoh et al (2002) demonstrated that a knockdown of *iro1* and *iro7* prevents formation of the isthmic organizer at the midbrain-hindbrain

boundary and it affects patterning in the forebrain and hindbrain. Loss of Wnt signaling leads to loss of *Irx* expression, whereas inhibition of Tcf3 activity (transcriptional repressor of Wnt target genes) leads to expansion of the *Irx* expression in forebrain tissue (Braun et al, 2003).

Expression patterns of *Six3* and *Irx3* are mutually exclusive. Misexpession studies by Kobayashi et al (2002) revealed that *Six3* can suppress *Irx3* expression, and reciprocally *Irx3* can suppress *Six3* transcription. Thus there are transcriptional mechanisms which allow formation of sharp borders within neuroepithelial domains. It is interesting that the posterior border of *Six3* expression regresses rostrally over time, making the diencephalic domain free from expression of both *Six3* and *Irx3* genes (Zuber et al, 2003).

It was found that the border between *Six3* and *Irx* expression lies where the zona limitans intrathalamica (zli) divides ventral and dorsal thalamus. The zli is marked by *Shh* expression, suggesting that the zli may be a signaling center (Zeltser et al, 2001).

Correct formation of the diencephalon, the eye, and the telencephalon depends on the proper inhibition of posteriorizing signals (Wnt and probably others) (Rallu et al, 2002). It was shown by Heisenberg et al (2001), in zebrafish, that inhibition of Wnt signaling is crucial for the subdivision of the forebrain into telencephalic, optic and diencephalic regions. A mutation of the Axin gene, a negative regulator of Wnt signaling, causes the masterblind mutation. In such mutants, the telencephalon is lost along with the eyes, and diencephalic tissue is expanded into these regions. A similar regional shift can be seen in mice which have inactive inhibitor of Wnt signaling *Six3*,

(Lagutin et al, 2003). Increasing the activity of Wnt antagonist Tlc within the anterior neural border leads to the shift of the telencephalic region to the optic field (Houart et al, 2002). It is possible to conclude that the eye field may require a level of activity of Wnt signaling lower than that needed for the development of prospective diencephalon and higher then that needed for the development of prospective telencephalon. It is worth mentioning that Wnt signaling blocks ventral telencephalic identity and is involved in the generation of telencephalic cells of dorsal character (Gunhaga et al, 2003). Lee et al (2000) established that multiple Wnt genes are expressed in the dorsocaudal edge of the dorsal telencephalon from embryonic day 8.5 and mice lacking Wnt3a activity in this field fail to develop the hippocampus.

Other signaling proteins expressed by the ANR are Fgfs. Fgf activity regulates regionalization of the telencephalon. Reduced Fgf signaling causes the following features: proliferation and apoptosis defects of the forebrain (Storm et al., 2003), midline defects (Walshe and Mason, 2003), and defects in morphogenesis of olfactory bulbs (Hebert et al, 2003). Both Fgf expression and susceptibility to Fgf may be regulated by Six3 and Irx signaling. It is interesting that expression of Fgf8 in the ANR depends on *Six3* activity (Lagutin et al, 2003), whereas *Fgf8* expression in the isthmus relies on *Irx* activity (Itoh et al, 2002). Moreover Fgf induces posterior markers in *Irx*-expressing regions and anterior markers in *Six3*-expressing regions (Kobayashi et al, 2002). Although Fgf seems to be expressed too late to be a primary inducer of the telencephalon, Fgf8 can restore expression of *Bf1* (*Foxg1*) in mouse explants of the neural plate deprived from the anterior neural ridge (Wilson and Rubenstein, 2000). In

telencephalon, Fgf activity may induce the polarization of cortical territories via repression of *Emx*2 (Garel et al, 2003), which reciprocally modulates Fgf activity. Thus Fgf promotes anterior cortical fates in the telencephalon.

1.5 Anterior neural border

Development of different regions within forebrain is promoted by the junction between anterior neural and non-neural ectoderm, the anterior neural ridge or border. This is an important structure, ablation of which leads to inability to express telencephalic markers, such as *Bf1* (*Foxg1*) and *Emx1*, possibly due to loss of Six3 expression (Lagutin et al, 2003). Also it was found that Tlc protein, expressed by anterior neural ridge cells, shows telencephalon inducing activity and is necessary for the establishment of forebrain identities (Houart et al, 2002). This protein belongs to the family of secreted Frizzled-related proteins (sFRPs) and acts as a Wnt-signalling antagonist, binding secreted Wnts (Uren et al, 2000).

1.6 Dorsoventral patterning of the telencephalon

Further development of the telencephalon includes dorsoventral patterning. There are two kinds of signaling which influence the dorsoventral polarity of the neural plate. The floor plate produces Sonic Hedgehog, Chordin and Noggin (the last two are Bmp antagonists), which ventralize the neural tube (Briscoe and Ericson, 2001). There

are two groups of dorsalizing signal produced by dorsal epidermal ectoderm (roof plate): Wnts and Tgf-β (include Bmps) (Lee et al, 1998) (Fig 1.2).

One of the important genes in this process is *Sonic Hedgehog* (*Shh*). It is needed for development of ventral neural structures. The earliest site of *Shh* expression appears in the midline mesoderm (Epstein et al., 1999). Then *Shh* mRNA appears in the primitive node. With closing of the neural tube the expression of *Shh* occurs in prechordal plate and anterior mesoderm. Just before neurogenesis, *Shh* expression starts in the hypothalamus, and then finally in the ventral telencephalon. *Shh* induces ventral telencephalic markers such as *Nkx2.1*, *Gsh2*, and *Dlx*2 (Corbin et al, 2003). The effect on the telencephalon of *Shh*-signalling is dependent on timing of expression rather than a concentration of *Shh*. Kohtz et al (1998) showed that *Shh* can not induce *Nkx2.1* (a ventral marker) expression beyond embryonic day 11.5.

It was established that Hedgehog signaling is mediated by *Gli* genes (Matise and Joyner, 1999). There are three *Gli* genes in vertebrates: *Gli1*, *Gli2*, and *Gli3,* which are zinc finger transcription factors. With lack of *Gli*1 and *Gli*2 gene functions, telencephalon develops relatively normally, whereas mutation in *Gli*3 leads to expansion of ventral markers into the cortex and loss of dorsal structures, such as hippocampus (Rallu at al, 2002). If *Shh* is mutated, then a dorsalized telencephalic phenotype is observed. If both *Shh* and *Gli*3 are mutated simultaneously the telencephalon develops a relatively normal dorsoventral patterning. These facts suggest that Shh and Gli3 have antagonistic actions and that this system is redundant for dorsoventral patterning of the telencephalon. Therefore there are *Shh* independent signaling pathways, which might act together to regionalize the telencephalon. Using conditional knockout of *Fgf* receptors in

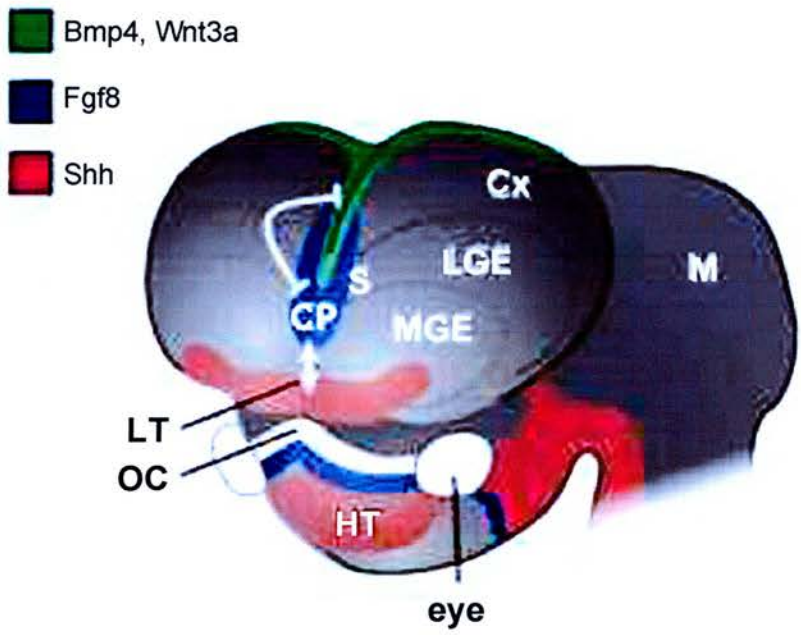


Fig 1.2. Patterning centers of a mouse brain. highlighting the patterning centers. White arrows- positive interactions; white lines with bars - repressive interactions; CP - commissural plate; Cx - Cortex; HT - hypothalamus; LT - lamina terminalis; LGE - lateral ganglionic eminence; MGE - medial ganglionic eminence; M - mesencephalon; OC - optic chiasm.(Modified from Sur and Rubenstain, 2005)

the mouse telencephalon Gutin et al. (2006) presented evidence that Fgf signaling is important for development of ventral telencephalon, as ventral precursors were not specified and all cells adopted dorsal fate despite *Shh* expression. Additional mutation of *Gli3* did not rescue the *Fgf* phenotype (unlike the *Shh* phenotype), which suggests that Fgf signaling is downstream of the Shh and Gli3 signaling pathway in determination of ventral fate.

It was established that in the absence of Shh full-length Gli3 slowly undergoes partial proteolysis to form a transcriptional repressor, which retains the DNA-binding domain but the C-terminal trans-activation region is removed, and that the presence of Shh suppresses Gli3 cleavage (Aza-Blanc et al., 1997, von Mering and Basler, 1999). Therefore regions of active Shh are free from repressor activity of Gli3, providing the opportunity for them to develop ventral (Shh) and dorsal (Gli3) telencephalon (Chiang et al., 1996; Theil et al., 1999). It is an interesting observation that the telencephalic midline is affected in *Shh, Gli3,* or *Fgf* mutants (Chiang et al., 1996; Theil et al., 1999; Storm et al., 2003). It was shown that Gli3 signaling is required for expression of Wnts and Bmp by dorsal midline (Grove et al., 1998; Theil et al., 1999). Also it is established that there is a dose dependent effect of Fgf8 on Bmp production in the dorsal midline (Storm et al., 2003). Gutin et al. (2006) proposed the following explanation: Fgf8 is suppressed by Gli3, which in turn is repressed by Shh. Therefore in *Shh* or *Fgf* mutants *Bmp* expression will be low and not enough to produce rostral midline because of Fgf deficit. In the case of Gli3 mutation dorsal telencephalic cells do not specify and Wnt expression is down-regulated or lost.

Gli3 mutants demonstrate different developmental defects of the forebrain: the telencephalon loses the choroid plexus, olfactory bulbs and cortical lamination are absent (Franz, 1994). Other researchers found that the telencephalic-diencephalic border is compromised leading to mixing of diencephalic and telencephalic cells (Fotaki et al., 2006). In the absence of Gli3 expression of dorsally localized genes such as *Emx1* and *Emx2* can not be induced or maintained (Theil et al., 1999). Also it was shown that ventricular zone organization, cortical progenitor apical/basal cell polarity and early born cortical differentiation are compromised in *Gli3* mutants (Theil, 2005).

Mutually exclusive gene expression is important to establish sharp boundaries between different areas of the telencephalon. The embryonic telencephalon can be subdivided into a dorsal pallium (which gives rise to the cerebral cortex) and a ventral subpallium (which gives rise to the basal ganglia). Future telencephalic territories can be defined early in development of this region by expression of a distinct set of homeodomain genes: *Nkx2.1*, *Gsh2* and *Pax6*. These mouse genes provide some of the earliest markers of dorsal (*Pax6*) (Stoykova et al, 2000), intermediate (*Gsh2*) (Yun et al, 2001) and ventral (*Nkx2.1*) (Sussel et al, 1999) parts of the telencephalon. Furthermore, these genes are crucial for normal development of these regions. Mice lacking *Pax6* express the subpallium genes in ventral pallium (Mash1, Dlx1) and MGE specific genes in LGE (Shh, Nkx2.1) (Stoykova et al, 2000). Mutation in *Gsh2* leads to expression of pallial markers in dorsal LGE (dorsalization) (Yun et al, 2001). A related phenotype is observed in mice with mutated *Nkx2.1*, i.e. ventral-to-dorsal transformation, in which

MGE cells transform to an LGE identity. The following interactions are described among these three genes: *Gsh2* and *Nkx2.1* seem to have no direct effect on each other's expression (loss of *Gsh2* does not lead to expansion of *Nkx2.1* expression and vice versa), *Gsh2* and *Pax6* repress each other, forming a sharp border at the LGE-cortex boundary (loss of *Gsh2* leads to expansion of *Pax6* expression to the LGE; mutations in *Pax6* drive *Gsh2* expression to *Pax6* domains) (Yun et al., 2001). Occupation of mutant gene territory by adjacent genes suggests that mutual repression is important to set boundaries of different regions with different properties in the telencephalon.

Besides the aforementioned genes there are bHLH (basic helix-loop-helix) transcription factors, which regulate neuronal identity. In the telencephalon, *Mash*1 is expressed ventrally (MGE, LGE), whereas two neurogenin (*Ngn*1, *Ngn*2) genes are expressed dorsally (dorsal telencephalon) (Schuurmans and Guillemot, 2002). The absence of either *Mash*1 or *Ngn*1/2 leads to defects in dorsoventral patterning, dorsalization in the case of *Mash*1 mutation and ventralization in the case of *Ngn*1/2 mutation. Homeodomain and bHLH proteins depend on each other. With the loss of *Gsh2*, expression of *Mash*1 is lost in the LGE, and expression of *Ngn*2 is expanded into this region. *Ngn*2 expression is lost in lateral cortex in *Pax6* mutants and *Mash1* expression expands into this region (Yun et al, 2001). In *Mash1*-null mice, MGE loses *Nkx2.1* expression (Casarosa et al, 1999). In *Ngn1/Ngn2* compound mutant mice, homeodomain (*Dlx*2) and bHLH (*Mash1*) genes which are expressed ventrally expand to dorsal regions. Additionally to dorsoventral patterning, *Mash*1 participates in proliferation of early ventricular zone progenitors and *Dlx*1/*Dlx*2 genes are required for

specification and differentiation of late progenitors in the same tissue as *Mash*1 (Yun et al, 2002). bHLH transcription factors are also expressed in mutually exclusive patterns.

As described above, *Emx2*, encoding a homeodomain protein, is one the genes with reduced dorsal expression in *Gli*3 mutants. Analysis of loss-of function mutation of *Emx*2 function leads to the loss of caudal-medial telencephalic domains, whereas rostral-lateral telencephalic domains expand (Bishop et al, 2000). An opposite shift occurs when *Pax6* is lost. Rostral-lateral domains where *Pax6* is expressed normally are lost because of the expansion of caudal domains where *Emx*2 is expressed (Bishop et al, 2000). It is possible that opposing activities of *Emx*2 and *Pax6* may generate graded positional identity within the dorsal telencephalon. Dorsal telencephalon progenitor cells become ventralized when they lose *Pax6* (Kroll and O'Leary, 2005). The first signs of ventralization are subpial and paraventricular ectopias which express markers of LGE but not of MGE. GABAergic not glutamatergic neurons are produced from transformed cells. The whole population of dorsal telencephalon progenitors becomes ventralized at mid-neurogenesis.

Bmp and Wnt signaling have their roles in pallium patterning. Several Bmps are expressed in and around the dorsal telencephalon. Ectopic activity of Bmp represses ventral telencephalic markers (*Nkx2.1*, *Dlx*2). Mutants lacking Bmp5 and Bmp7 have delayed closure of the rostral neural tube, hypoplasia of the telencephalic vesicles, and reduced apoptosis in the telencephalic roof (Solloway et al, 1999). Mutations in *Wnt3a* and in a downstream effector of the Wnt pathway, Lef1, lead to reduction of the size of

the hippocampus, because of reduced proliferation in the hippocampal anlage (Lee et all., 2000; Galceran et al,.2000), indicating that Wnt signaling is required for development of this structure.

Fig 1.3 shows the further partition of the dorsal telencephalon into the medial pallium (MP), which is the source of hippocampus; dorsal pallium (DP), which is the anlage of the neocortex; the lateral pallium (LP), which gives rise to olfactory cortex; the ventral pallium (VP), from which the claustroamygdaloid complex is generated; and ventral telencephalon, which encompasses: lateral ganglionic eminence (LGE), which generates striatum, and medial ganglionic eminence (MGE), which gives rise to the pallidum. All these regions are different in their gene expression patterns. There is further subdivision of LGE to ventral LGE and dorsal LGE, which have different levels of *Pax6*, *Gsh2*, *Dlx*2, and *Mash*1. All these genes have higher levels of expression in dLGE. Gsh1 is expressed in vLGE only.

1.7 Origin of Telencephalic Neurons

Many mechanisms participate in patterning of the cerebral cortex and basal ganglia. It seems that different parts of the telencephalon develop independently. However there is interdependence among these regions. In dorsal telencephalon there are three ganglionic eminences: MGE (medial ganglionic eminence), LGE (lateral ganglionic eminence), and CGE (caudal ganglionic eminence), from which cells migrate to the cortex. Different regions are sources of different types of neurons. MGE and CGE

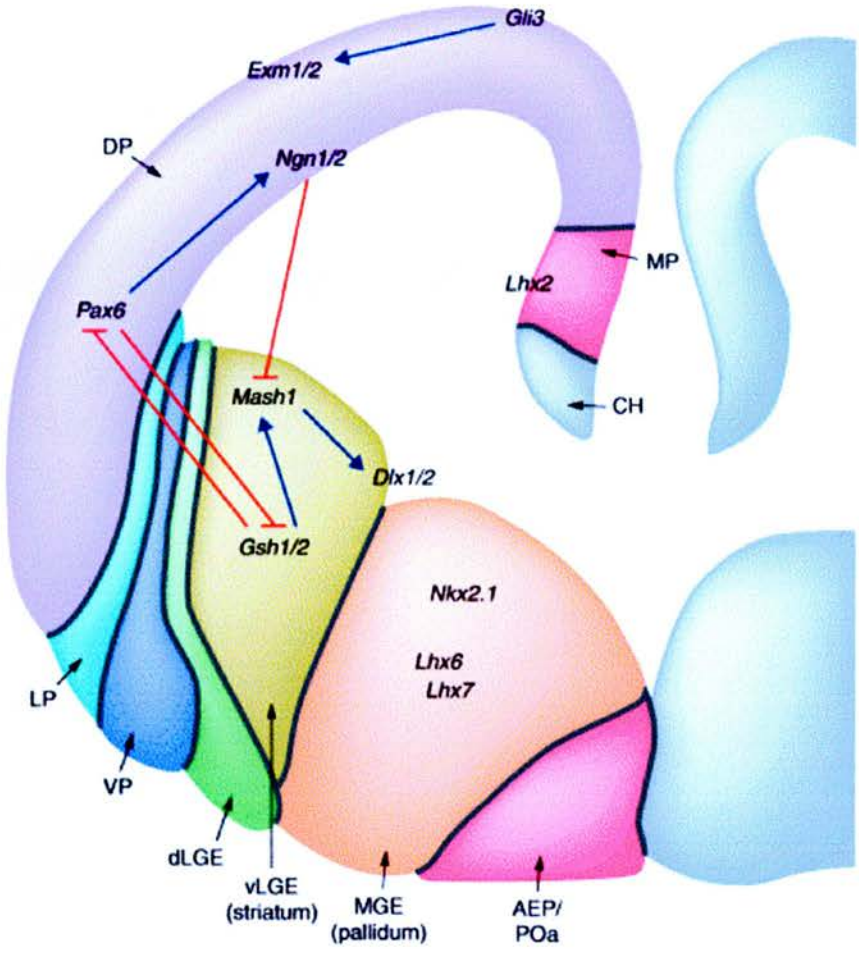


Fig 1.3. Genetic interaction in regionalization of the mammalian telencephalon. Abbreviation the same as in text, plus AEP/POa (anterior entopeduncular/preoptic area) and CH (cortical hem). Genes expressed in particular areas are shown. Arrows denote positive interaction; T-bars denote inhibitory control. (from Schuurmans and Guillemot , 2002)

generate populations of interneurons and oligodendrocytes; whereas the LGE, cortical and hippocampal proliferative zones produce primarily projection neurons (Marin and Rubenstein, 2001).

Also it is possible to trace the origin of neurons by the type of neurotransmitter they express: GABAergic neurons are produced in MGE and LGE; cholinergic neurons are derived from MGE; glutamatergic neurons emerge in the cortex. Interestingly, cholinergic projection neurons stay in the MGE, whereas cholinergic interneurons migrate to the LGE/striatum (Marin et al, 2000). The main pathways of tangential migration are presented in Fig 1.4.

1.8 Cell division types of telencephalic neuronal progenitors

Neuronal progenitors in the developing cortex can undergo different types of cell division. Divisions can be symmetric or asymmetric. In the case of symmetric division two daughter cells are equal whereas in asymmetric division daughter cells are different. An additional characteristic of the type of division is whether daughter cells are equal to the mother cell. By this point of view a division can be differentiative (daughter cells are different from the original cell) or proliferative (daughter cells have similar potential to proliferate as the mother cell). Also there are mixed variants when one daughter cell is the same as the parent but the other is not and two daughter cells are different from each other and the mother cells. Progenitors involved in neurogenesis are neuroepithelial cells, radial glial cells, and basal (or intermediate) progenitor cells. Basal progenitor cells are cells which migrate to the basal boundary of the ventricular zone or

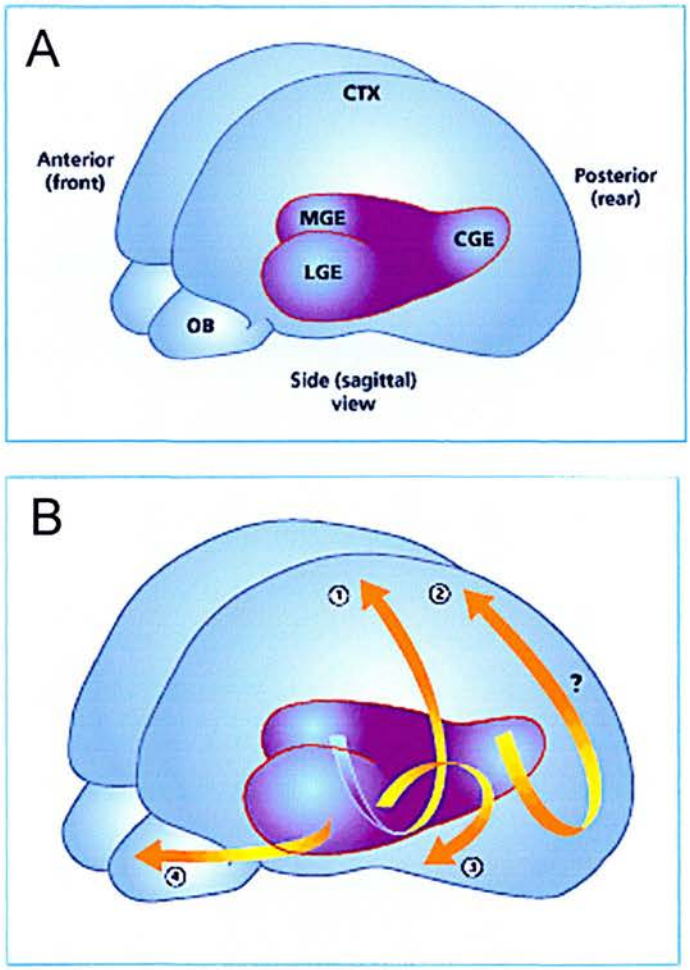


Fig 1.4. A) **The basic anatomy of the embryonic telencephalon**. MGE (medial ganglionic eminence), LGE (lateral ganglionic eminence), CGE (caudal ganglionic eminence), CTX (cerebral cortex), and OB (olfactory bulbs). B) **Routes of tangential migration**: 1 –from MGE to the dorsal telencephalon, 2- from CGE to the dorsal telencephalon, 3 – from the cortical striatal boundary to the ventral telencephalon, 4 – from LGE to the olfactory bulb (rostral migratory stream). The question mark on arrow (2) indicates that this route is less well characterized than the others (from Corbin et al., 2001).

subventricular zone to undergo S phase and mitosis (reviewed in Gotz and Huttner, 2005). Basal progenitors retract their processes from the apical surface (Fig.1.5).

Neuroepithelial cells are the primary progenitor cells. These cells give rise to all progenitors and –directly or indirectly –neurons in the central nervous system (Huttner and Kosodo, 2005). Neuroepithelial cells increase their number by symmetric proliferative divisions, when the neuroepithelial cell divides into two neuroepithelial cells. Also these cells can generate radial glial cells, but the mode of division is still not clear. Neurons are generated from neuroepithelial cells by asymmetric monoproliferative division (one daughter cell is a neuroepithelial cell and the other is a neuron) or by symmetric differentiative division when the two new cells are neurons (Saito et al, 2003). The fact that basal progenitor cells appear before radial glial cells proves that neuroepithelial cells are progenitors for basal progenitor cells as well (Haubensak et al, 2004). However, the mechanism of this has to be elucidated.

The radial glial cell pool amplifies by symmetric proliferative division. Asymmetric monodifferentiative division of radial glial cells produces basal progenitor cells (Noctor et al, 2004) or neurons (Miyata et al, 2001). The result of symmetrical differentiative radial glial cell division is two neurons (Miyata et al, 2004).

Basal progenitor cells undergo two kinds of division. First is symmetric proliferative and the second is symmetric differentiative when only neurons are generated (Noctor et al, 2004).

Changes in the size or cycling rate of the proliferative pool could have various effects on telencephalic development (Chenn and Walsh, 2002; Martynoga et al., 2005).

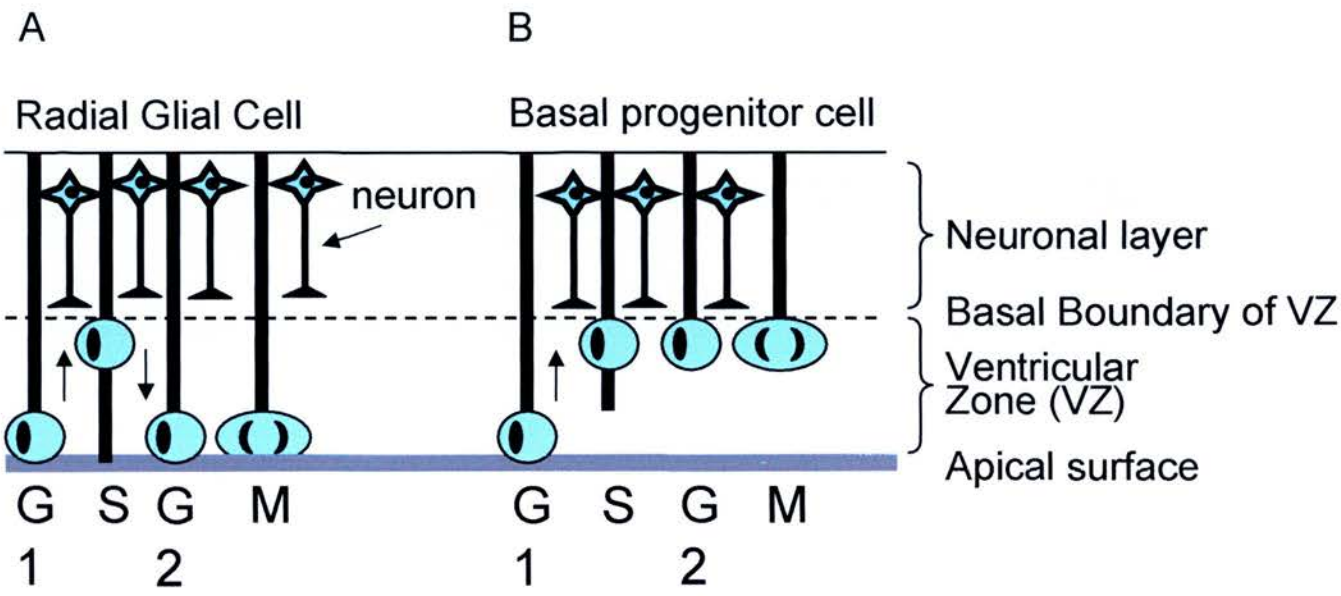


Fig 1.5. Differences between the radial glial and basal progenitor cells.
A. The nucleus of the radial glial cell migrates to the basal surface of the ventricular zone (VZ) where S phase happens.
Then nucleus moves back to the apical surface where mitosis occur.
B. The basal progenitor cell migrates to the basal boundary of the VZ where it undergoes S phase and mitosis. Apical
process is retracted.

1.9 Differentiation of neural cell progenitors: neurons versus glia

Neural cell progenitors differentiate into glial cells or neuronal cells. What are the factors which drive the decisions of progenitor cells? Extracellular factors, such as Bmp2, fibroblast growth factor2 (Fgf2) and leukemia inhibitory factor (Lif) participate in glial fate decision. It was found that some of the downstream effectors of these signaling molecules have a dual role: they promote glial fate and inhibit neurogenic differentiation. As an example: Bmp2 with its downstream effector Smad1 and Lif with its downstream effector STAT3 form a gliogenic transcriptional complex, which consists of Smad1/STAT3/p300 and promotes astocytic differentiation. Also Bmp2/Smads induce expression of Hes5, which interferes with Ngn1, a neurogenesis-inducing protein (Nakashima et al, 1999). Ngn1 has an opposite dual activity: it promotes neurogenesis through transcriptional activation of neurogenesis genes and suppresses gliogenesis by combining with Smad/p300 counteracting formation of gliogenic Smad1/STAT3/p300 complex (Sun et al, 2001). Other mediators, which promote neuronal fate, are *Mash*1 and *Ngn*2. The bHLH (basic helix-loop-helix) gene *Ngn*2 seems to be a permissive co-factor in specification of neural identity, acting in combination with as yet unknown instructive factors. Parras et al (2002) used a knock-in model where *Mash*1 replaced *Ngn*2 or *Ngn*2 replaced *Mash*1 in the expression domain specific to the replaced gene. They showed that *Mash*1 can respecify a variety of distinct neuronal lineages when ectopically expressed in precursors that would normally express *Ngn2*, whereas *Ngn*2

cannot respecify progenitors that would normally express *Mash1*. This indicates that *Mash*1 has an instructive role in specifying the identity of various neuronal subtypes whereas the role of *Ngn*2 in this process is permissive.

The predominant type of neurons found in the cortex is glutamatergic projection neurons, which originate from dorsal telencephalic progenitors, and interneurons, which originate ventrally. Cortical projection neurons can be recognized by expression of *Tbr1* and bHLH proteins (NeuroD, NeuroD2, Math2 and Math3). bHLH factors play an important role in differentiating telencephalon: NeuroD and Math2 mutants show differentiation defects in the dentate gyrus (Liu et al, 2000). *Lhx5* is a neuronal differentiation gene: it is required for proper proliferation and differentiation of hippocampal precursors but not for initial specification.

Specification of GABAergic interneurons depends on the operation of the following genetic pathway: *Mash*1→*Dlx*→*GAD*. This pathway is active in the ventral telencephalon. *Mash*1 induces expression of *Dlx* genes, which induce biosynthesis of glutamic acid decarboxylase (GAD). GAD regulates a rate-limiting step in GABA synthesis (Anderson et al, 1999). It is possible to distinguish GABA interneurons born in the MGE or in the LGE. Interneurons from the MGE express *Nkx2.1* and *Lhx*6/7 (Marin et al, 2000).

Neural stem cells can differentiate into oligodendrocytes also. Oligodendrocytes are among the latest differentiating cells in the nervous system, although oligodendrocyte precursors (OLPs) are specified in early embryonic stages. Experiments on quail-chick chimeras showed that the telencephalic site of origin of oligodendrocyte precursors is the anterior entopeduncular area (AEP). OLPs express platelet-derived

growth factor and migrate from AEP to MGE, LGE and cortex. Sonic hedgehog can induce oligodendrocyte development in ventral regions of the nervous system, including the telencephalon (Nery et al, 2001). In response to *Shh*, two novel bHLH factors (*Olig*1 and *Olig*2) are expressed. Furthermore, overexpression of *Id*2, a negative regulator of bHLH activity, suppresses oligodendrocyte development. This suggests that bHLH factors are involved in oligodendrocyte specification.

It is worth mentioning that GABAergic neurons and oligodendrocytes in the dorsal telencephalon originate from common progenitors located in the ventral telencephalon (Monuki and Porter, 2001).

The important characteristic of neural stem cells (NSCs) is maintaining a pluripotent state. Several genes participate in this process. *Hes* (Hairy/Enhancer of Slit, belong to bHLH family) genes downregulate the activity of bHLH transcription factors that are required for neuronal differentiation. Mutants for *Hes1/Hes5* exhibit reduced self-renewal capacity of stem cells and ectopic expression of these genes postpones differentiation of the telencephalic progenitors both to neurons and glia (Ohtsuka et al, 2001). The membrane protein Notch controls *Hes*1/5 expression and inhibits neurogenesis (Solecki et al, 2001). A summary of neural stem cell fates and factors which influence fate decision is in Fig 1.6 (from Schuurmans and Guillemot, 2002).

1.10 The cerebral cortex: lamination

The cerebral cortex is a six-layered structure. The development of these neuronal layers involves the migration of neurons in radial and tangential directions to their final

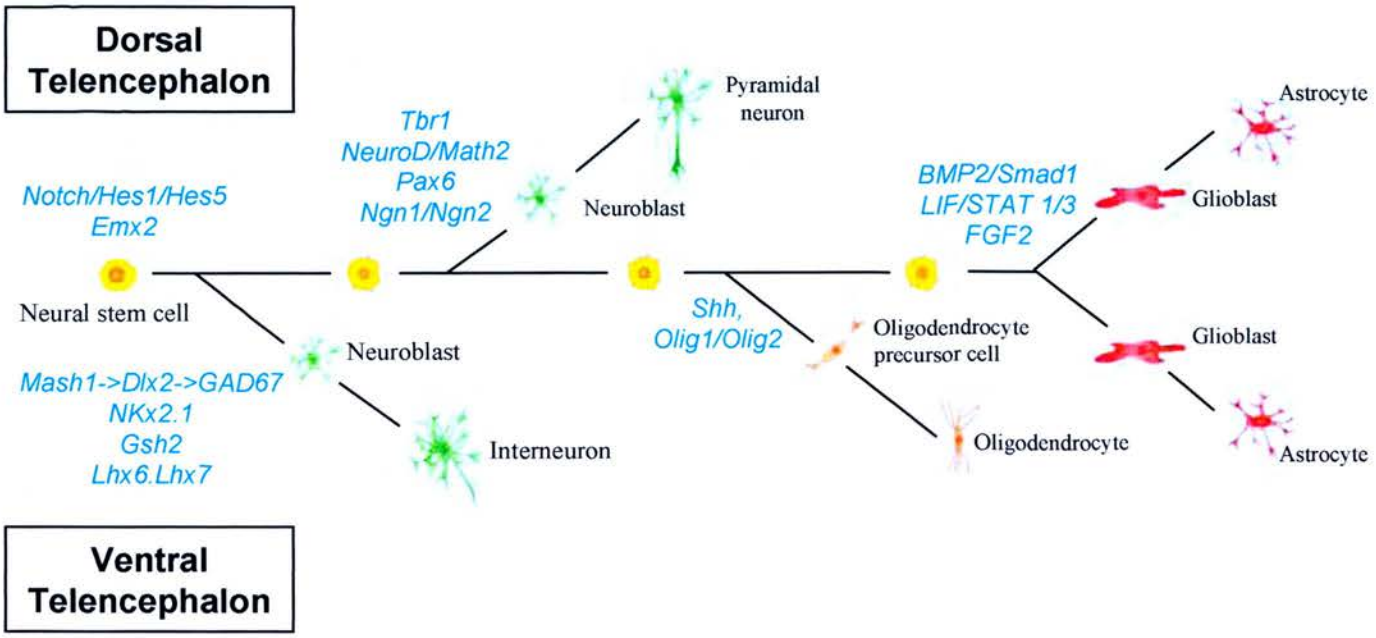


Fig 1.6. Summary of factors involved in specification of different neuronal and glial fates in the dorsal and ventral telencephalon. Notch/Hes1/Hes5 maintain multipotent status of NSC; Mash1-Dlx2-GAD67, Gsh2, Lhx6/Lhx7 promote GABAergic specification; Tbr1, NeuroD/Math2, Ngn1/Ngn2 stimulate differentiation of pyramidal neurons; Shh, Olig1/Olig2 promote development of oligodendrocytes; BMP2/Smad1, LIF/STAT1/3, FGF2 lead to differentiation to astrocytes (modified from Schuurmans et al., 2002)

laminar positions. In the first stage the cortical anlage consists of two layers: the ventricular zone (VZ, proliferative zone) and preplate. In the next stage postmitotic neurons exit the VZ and migrate radially toward the pial surface, splitting the preplate into the subplate (below, closer to the VZ) and the marginal zone (above, farther from the VZ). The result is formation of the cortical plate which is established in between the subplate and marginal zone. Layers 2-6 of the cortex develop when neurons migrate from proliferative zones into the cortical plate. Neurons from proliferative zones of the MGE and LGE migrate into the cortex tangentially and then radially to find the appropriate layer (Marin and Rubenstein. 2003).

Radial migration establishes the general cytoarchitectonic framework of the different forebrain subdivisions (Marin and Rubenstein, 2003). During this type of migration cells move from the VZ toward the marginal zone of the cortex. The necessary condition for proper migration is formation of a link between the ventricular zone and the pial surface by radial glial cells. In general the soma of a radial glial cell lies in the VZ, while a process reaches the pial surface. One function of radial glial cells is to provide static supportive elements along which neuronal precursors migrate (Miyata et al, 2001). After completion of neuronal migration, radial glial cells retract their ventricular and pial attachments and differentiate into astrocytes (Marin-Padilla 1995; Hunter and Hatter al., 1995). Also radial glial cells can differentiate into neurons (Malatesta et al. 2000). The transition from radial glia to neurons can go in this sequence: radial glia (express *Pax6*) → intermediate progenitor cells (express *Tbr*2) → postmitotic neurons (express *Tbr*1). There are two types of radial migration: locomotion and nuclear translocation. Locomotion is a glia-guided process and involves movement

of the whole cell including its leading and trailing processes. Nuclear translocation (nucleokinesis, somal translocation) appears to be largely independent of radial glial cells (Nadarajah et al. 2001). During nucleokinesis the cell stretches the leading process which ends at the pial surface. The nucleus migrates through the elongated process to its destination.

There are a few kinds of tangential migration. In one case, neurons migrate in a group using each other to promote their migration (migration of olfactory bulb interneuron precursors). In another case, neurons follow growing axons to reach their destination (migration of neurons producing gonadotropin releasing hormones). Also some neurons may not follow specific cellular substrates and may disperse in an individual manner (migration of interneurons and oligodendrocytes from the subpallium to the cortex) (Marin and Rubenstein, 2003).

It was established that cells of deeper layers of the cortical plate become postmitotic earlier than those of the more superficial layers. Proper layer formation depends greatly on interaction between migrating neurons and Cajal-Retzius cells. Cajal-Retzius cells are born at E10.5 – E11.5, probably originate in the MGE and/or the cortical hem (Hevner et al, 2002), and migrate to the marginal zone of the cortex. Migrating neurons pass over previously generated neurons until they reach the marginal zone. Then neurons detach from the radial glia. This suggests that layer formation in the cortex relies on interaction between migrating neurons and marginal zone cells (Cajal-Retzius cells). Cajal-Retzius cells express Reelin (a large secreted protein which associates with the extracellular matrix), whose mutation leads to abnormalities in

neuronal migration. In mice with such a mutation (reeler mice) the first wave of migrating neurons fails to split the preplate. The next waves of neurons are unable to pass the previous ones forming an inverted direction of layer formation in the cortex. There are three hypotheses as to how Reelin participates in laminar positioning of migrating cells in the cortex. One is that Reelin restrains neuronal migration. Indeed, exposure to recombinant Reelin reduces the rate of neuron migration (Dulabon et al. 2000). However Magdaleno et al (2002) demonstrated that its ectopic expression in the ventricular zone of the cortex does not prevent radial migration. The second is based on observations that a normal glial scaffold fails to form in the dentate gyrus of the hippocampus of reeler mice (Frotscher et al., 2003) and removal of Cajal-Retzius cells, producers of Reelin, stimulates premature differentiation of radial glial cells into astrocytes (Super et al. 2000). These findings suggest that Reelin can regulate radial glial identity, functioning and consequently their ability to position neurons. The last is that Reelin enables detachment of neurons from radial glia (Dulabon et al. 2000). Neurons born from the same radial progenitor cell use the same radial process to reach the cortical plate. Therefore a lack in detachment of earlier migrating neurons can form a physical obstacle for the following waves of migration. These hypotheses are not mutually exclusive.

Another signaling pathway that is known to control layer position during development involves cyclin-dependent kinase 5 (Cdk5) and its activating subunits, p35 and p39. Defects similar but not identical to those in Reelin mutants are observed in mice lacking Cdk5, or p35, or both p35 and p39. Mutations in the Cdk5 signaling

pathway exhibit a cortical inversion as in reeler mice, but do not prevent splitting of the preplate into the marginal zone and subplate (McEvilly et al. 2002).

There are genes which mark the laminar identity of the cortex. *Pax6* is expressed in the ventricular zone and marks radial glial cells. In *Pax6* mutants the SVZ is decreased and fewer upper layer neurons are produced (Heins et al, 2002). *Tbr*2 is expressed strongly in the subventricular zone (SVZ) and weaker in the basal VZ and lower intermediate zone (IZ). *Tbr*2 marks intermediate progenitor cells. *Tbr*1 has a role in the generation of defined subpopulations of cortical neurons. This gene is expressed at a high level in early born glutamatergic neurons of the marginal zone, cortical plate, subplate, and at a lower level in the intermediate zone. The cortex of *Tbr*1 mutants displays developmental abnormalities in the laminar organization of neurons, and in the guidance of cortical afferent and efferent axons (Hevner et al, 2001).

1.11 Adenomatous Polyposis Coli

1.11.1 General description of Apc

APC (*Adenomatous Polyposis coli*) was originally identified as the gene mutated in Familial Adenomatous Polyposis (FAP). FAP is a gastrointestinal tract disease which is characterized by formation of multiple colorectal adenomas and is inherited as an autosomal dominant trait assigned to chromosome 5q21 in humans (Stella et al., 1992). *Apc* encodes a protein which was discovered in colorectal cancers (Nathke 1999). This

protein has 2842 amino acids. Its weight is 310kDa. Apc localizes in the cytoplasm and nucleus. The *Apc* gene comprises 16 exons.

There are three parts in Apc: N-terminus, M (middle)-part, and C-terminus. All these parts have different functions (Nathke, 2004) (Fig 1.7).

The N-terminus contains an oligomerization domain, nuclear export signal (NES) domains, and armadillo repeats.

The oligomerization domain is important for dimerization of Apc protein. Nuclear export signal domains are needed for shuttling Apc between the nucleus and cytoplasm. Also the NES domain interacts with Crm-1 exportin. Armadillo repeats are responsible for interaction of Apc with the following proteins: Asef – Apc-stimulated guanine nucleotide exchange factor for Rho family proteins, IQGAP1 – Rho GTPase effector, Kap3 – a linker protein for kinesins, and regulatory subunit of protein phosphotase 2a (PP2a) (Hanson and Miller,2005).

The middle part of Apc contains two types of sites which can bind β-catenin: the 15 amino acid repeats constitutively bind β-catenin, and the 20 amino acid repeats whose ability to bind β-catenin is regulated by their phosphorylation. Plakoglobin (γ-catenin) binds these repeats as well. Axin binds to SAMP repeats of the middle region of Apc (Nathke, 2004a).

The C-terminus of Apc protein binds microtubules, EB1 (small microtubule end binding protein), PDZ domain containing proteins, and mDia (scaffolding protein).

An overview of Apc protein domains is shown in Fig 1.7.

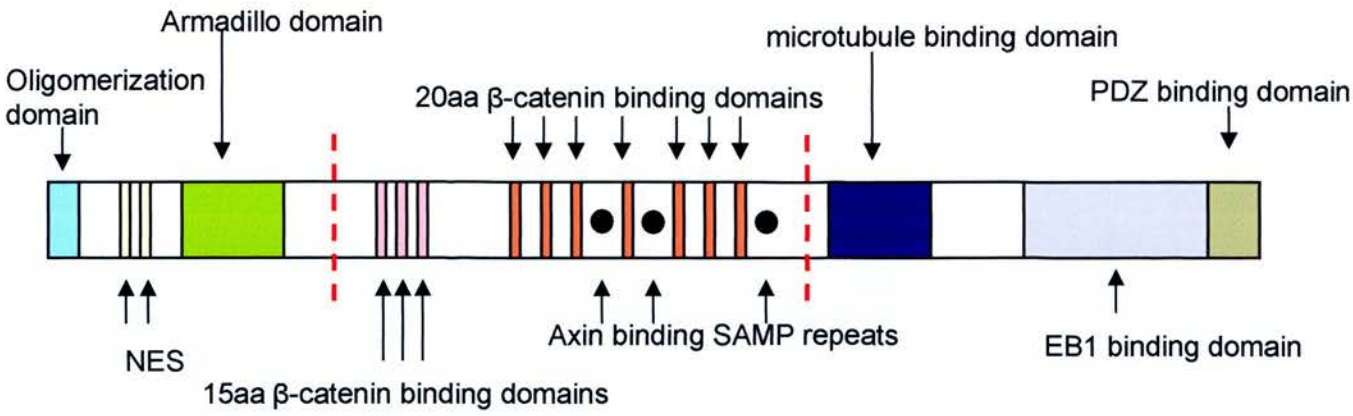


Fig 1.7. Schematic structure of APC protein. NES –nuclear export signal domains, aa – amino acids. Red dashed lines separate from left to right: N-terminus, M-part, and C-terminus of Apc protein

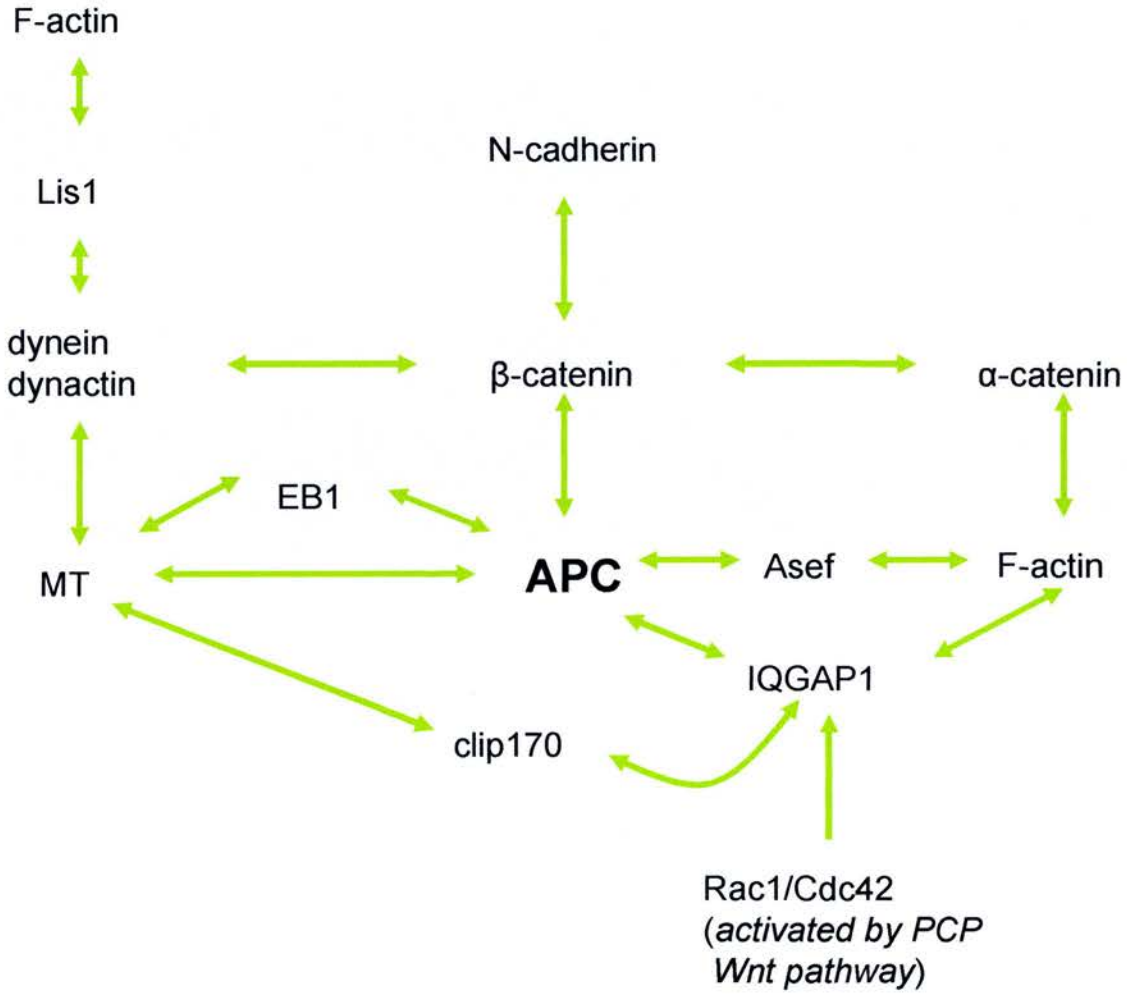


Fig 1.8. Interaction of APC in the cytoskeleton. Interaction of protein in the cytoskeleton is shown. Double leaded arrows show physical interactions between proteins.

1.11.2 Apc functions

The very large number of known interacting partners for Apc suggests that this protein is involved in many cellular processes. Indeed Apc has been shown to influence: cell-cell adhesion, cell migration, cell cycle progression, cell death (Hanson et al, 2005), neuronal differentiation, Wnt signaling pathways (Votin et al 2005), mitotic spindle assembly and chromosome segregation (Green et al, 2005).

1.11.3 Apc and the cytoskeleton

There are a few ways in which Apc is involved in cytoskeletal organization. Fig 1.8 presents a schematic picture of known Apc interactions with cytoskeletal proteins.

Apc interacts with cytoskeletal β-catenin. Apc competes with N-cadherin for β-catenin because Apc and N-cadherin bind the same region of β-catenin. Apc and N-cadherin neither interact with each other nor associate in one complex with β-catenin. This kind of interaction can affect N-cadherin adhesion by changing the level of available β-catenin (Klingelhofer et al., 2003).

Apc interacts with Rho GTPase effectors providing a link between the cytoskeleton and Rho GTPases. Asef, a Rac specific GEP (guanine nucleotide exchange factor), is activated by Apc. This results in a decrease of cadherin mediated adhesion and increased cell migration and lamellipodia formation (Kawasaki et al., 2003).

IQGAP1 is another Rho GTPase effector. It contains an IQ motif which binds calmodulin. IQGAP1 interacts with Apc at the leading edge of the cell regulating cell adhesion and migration. Apc-IQGAP complex also includes Clip-170 protein, which

interacts with microtubules, and activated Rac1/Cdc42 (Rho GTPases). As IQGAP in complex with Apc binds actin it can provide a link strong between the actin cytoskeleton and microtubules through the ability of Apc to bind microtubules. The presence of CLIP-170 bound to IQGAP1 in this complex provides an additional link to microtubules. Apc through Asef can activate Rac1. Activated Rac1 affects microtubules and the actin cytoskeleton through binding with IQGAP1. Either IQGAP1 or Apc depletion inhibits polymerization of actin filaments at the leading edge of the cell. This decreases cell migration and immobilization of the plus-ends of microtubules (Noritake et al., 2005).

An additional pathway linking Rho GTPase to Apc and the cytoskeleton includes mDia scaffold protein. Binding of active Rho relieves the autoinhibition of mDia then mDia, Apc and EB1 protein associate in one complex. This complex captures and stabilizes microtubules at the plus end (Wen et al., 2004).

The C-terminus of Apc contains a basic domain which binds the plus end of microtubules, leading to increased microtubule stability. Also Apc binds non-assembled tubulin and promotes the assembly of branched microtubule arrays (Zumbrunn et al., 2001). If the microtubule binding domain of Apc is lost cells show decreased ability to migrate.

EB1 protein binds the plus-end of microtubules when bound to Apc EB1 binding domain and promotes microtubule polymerization and thus cell migration (Wen et al., 2004).

Interaction of Apc with KAP3 protein (kinesin-superfamily associated protein 3) provides a link to KIF3A and KIF3B proteins of the kinesin superfamily, microtubule

plus-end-directed motor proteins. This interaction is important for cell migration by accumulation of Apc and β-catenin at the leading edge of the cell or at the tip of membrane protrusions (Jimbo et al., 2002).

1.11.4 Apc and mitotic spindle assembly and chromosome segregation

A strong interaction of Apc and EB1 proteins was found at the end of the plus tip of microtubules. This fact suggests that these proteins can play a pivotal role in associating microtubules with kinetochore (Berrueta et al., 1998, Dikovskaya et al., 2004). Experiments in Drosophila (Yamashita et al., 2003) showed that Apc is important in the orientation of the mitotic spindle during asymmetrical mitosis because spindle orientation and centrosome position are disturbed in Apc mutant germ cells.

There is evidence that colon cancer cells with mutated Apc experience chromosome instability and aneuploidy as a result of this (Nowak et al., 2002). This suggested a hypothesis that Apc is involved in proper chromosome segregation. Interestingly, inhibition of Apc or EB1 leads to chromosome alignment defects without mitotic arrest, whereas mutations of other plus end microtubule binding proteins (Lis1, dynein, CLIP 170) also cause chromosome alignment defects but with robust mitotic arrest (Green et al., 2005). As Apc interacts with the plus end of microtubules and localizes at the kinetochore it can mediate proper attachment of microtubules to the kinetochore thus ensuring proper chromosome segregation (Dikovskaya et al., 2004). Bub (Bub1 and Bub3) checkpoint kinases interact with Apc during mitosis facilitating

the proper attachment of microtubules to the kinetochore (Kaplan et al., 2001). Bub kinases are present at the kinetochore early in mitosis, decrease when microtubules attach at metaphase, and completely dissociate from the kinetochore by anaphase. In Apc mutant cells the level of Bub kinases at the kinetochore does not change at metaphase, but it diminishes by anaphase (Green and Kaplan, 2003). An increase in Bub kinases level in Apc mutant cells suggests an interaction of these two proteins which can influence chromosome stability.

1.11.5 Apc and neuronal differentiation

Apc is highly expressed in the central nervous system of vertebrates and invertebrates (Brakeman et al., 1999). An increase of Apc level in neuronal cell culture in response to nerve growth factor (NGF) suggests involvement of Apc in neuronal differentiation (Zhou et al., 2004). Also it was shown in pheochromocytoma PC12 cells that suppression of Apc by antisense mRNA prevents neuronal differentiation of these cells induced by NGF. However Apc suppression had no effect on the morphology of these cells once they differentiated (Dobashi et al., 2000). Zhou et al., (2004) showed that for rapid axon growth PI3K (phosphatidylinositol 3-kinase) should be activated at the growth cone. This PIK3K activation leads to inactivation of Gsk3β by its phosphorylation. As a result Apc is no longer phosphorylated by Gsk3β and therefore can bind microtubules and promote axon growth. PI3K is activated in response to NGF.

A pathway which also affects Apc and neural differentiation in a similar way includes Cdc42, Par6 and PKCζ. Par6-PKCζ complex can phosphorylate Gsk3β when Cdc42 is activated. Cdc42-dependent phosphorylation of Gsk-3beta promotes the

interaction of Apc with the plus ends of microtubules (Etienne-Manneville and Hall, 2003).

1.11.6 Apc and the cell cycle

Ishidate et al. (2000) showed that Apc binds hDLG (human Disk Large) by PDZ domain. This complex regulates G_1/S transition. Overexpression of wild type Apc can block G_1/S transition whereas overexpression of Apc without hDLG binding domains has a lower cell cycle blocking activity. Also the block of cell cycle progression by wild type Apc can be because of negative regulation of c-Myc and CyclinD1 expression by Apc.

It was found that mutation of Apc in different cancers correlates with a decrease in apoptosis (Venesio et al, 2003). Also Browne et al. (1998) demonstrated that Apc is cleaved by caspases during apoptosis. Apc can promote apoptosis in several ways. Anti-apoptotic protein Survivin is down-regulated by Apc (Zhang et al., 2001). Another protein with pro-apoptotic activity is Clusterin. This protein is up-regulated by expression of full length Apc and down regulated in tumors with mutated Apc (Chen et al, 2004).

1.11.7 Apc and Wnt signaling

Wnt signaling is a highly evolutionary conserved signaling pathway (Cadigan and Nusse, 1997). Wnt molecules are secreted palmitoylated glycoproteins which are

crucial for intercellular communication (Willert et al., 2003). Wnt signaling is involved in many cell processes such as cell migration, proliferation, cell death, and cell polarity (Olson and Parcoff, 1994; Bournat et al., 2000; Sasakura et al., 1998). There are three Wnt signaling pathways: canonical, planar cell polarity and Wnt/calcium (Nusse, 1997; Moon et al., 1997; Slusarski et al., 1997). Schematic representations of all three Wnt pathways can be found in Fig 1.9.

Canonical Wnt signaling includes activation of cytoplasmic scaffold phosphoprotein Dishevelled (DVL) through interaction of Wnt molecules with Frizzled receptors and Lrp5/6 (low-density lipoprotein receptor related protein) (Mao et al., 2001). When DVL is activated it promotes disassembly of Axin/Gsk-3β/Apc/β-catenin complex (Zeng et al., 1997). In the absence of Wnt signal this complex phosphorylates β-catenin thus promoting its degradation (Papkoff et al., 1996). When this complex is disassembled by DVL, β-catenin is no longer phosphorylated and β-catenin levels in the cytoplasm increase. Beta-catenin translocates to the nucleus where it forms a complex with Tcf/Lef (Korinek et al., 1997). This complex activates transcription of target genes (e.g. *c-myc*, *CyclinD1*, *fibronectin*, *Bmp-4*, *mab-5*, c-jun, Tcf1, Lef1 and many others) (Logan and Nusse, 2004, Nusse 2005). In addition to promotion of beta-catenin destruction Apc can suppress beta-catenin mediated transcription of target genes in two other ways (Xiong and Kotake, 2006). The first option is that Apc promotes beta-catenin export from the nucleus. The second way is that Apc binds beta-catenin on the chromatin that blocks transcription of Wnt target genes (Sierra et al, 2006).

The planar cell polarity pathway starts with activation of DVL through Frizzled by Wnt ligands. This pathway does not need Lrp5/6 for activation. In this case DVL

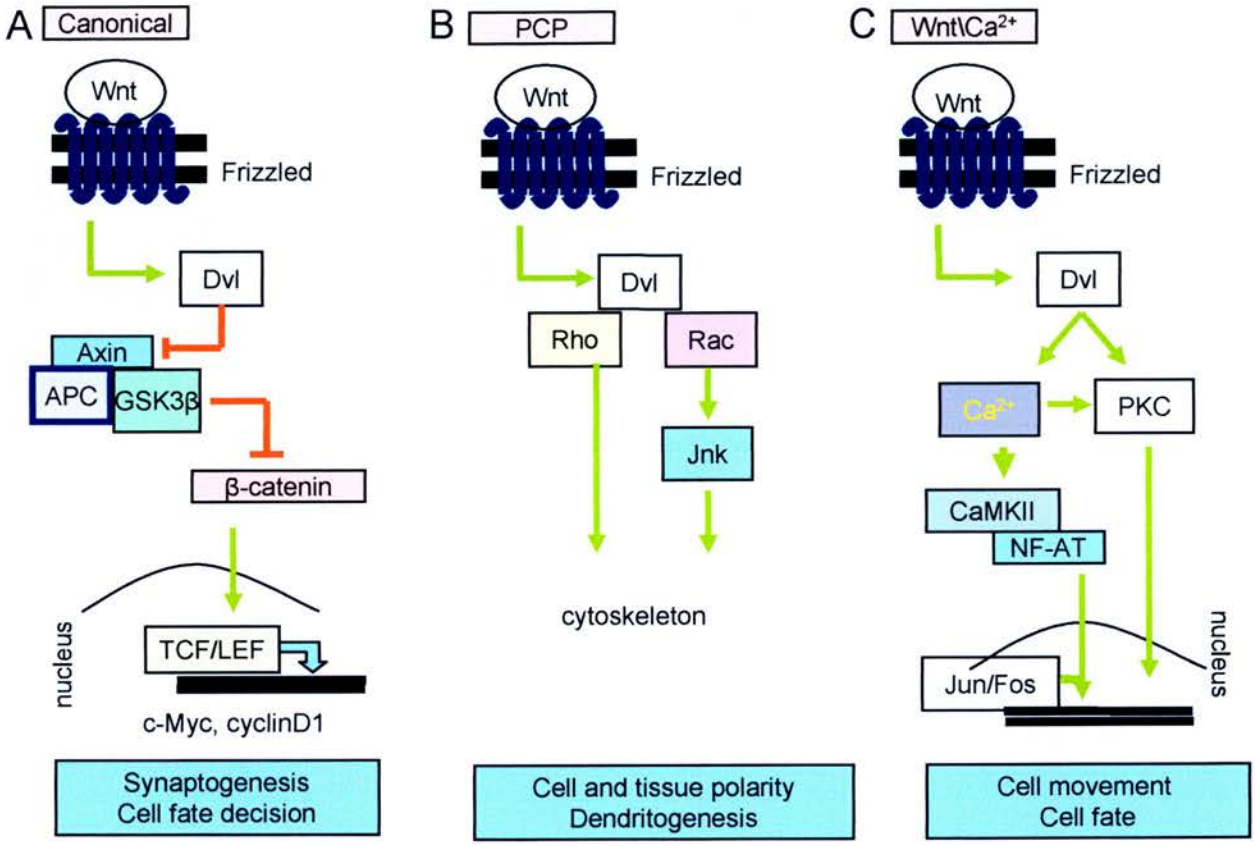


Fig 1.9. Three WNT pathways: a) canonical Wnt signalling pathway. Binding Wnt to Frizzled activates Dvl. Activation of Dvl inhibits Gsk3β, which leads to accumulation of β-catenin and its translocation to the nucleus where it activates transcription. **b)** Planar cell polarity (PCP) pathway. Binding Wnt to Frizzled activates Dvl. Activation of Dvl activates Rac and/or Rho. This promotes cytoskeleton changes. **c)** Wnt/Ca^{2+} pathway. In response to Dvl activation intracellular calcium is released and PKC activated. This activates CaMKII. NF-AT moves to the nucleus where activates transcription.

activates small GTPases of the Rho family – RhoA, Rac1, JNK and possibly others (Habas et al, 2003). Noritake et al, (2005) clarified the influence of PCP pathway on microtubules and actin cytoskeleton. Rac1 and Cdc42, another Rho-GTPase, interact with IQGAP, when activated. Then IQGAP interacts with Apc and Clip-170, a microtubule binding protein. Also IQGAP can directly interact with actin. Interaction of IQGAP and Apc increases microtubule stability and provides a strong link between microtubules and actin filaments. Another link is provided by Rho. It activates scaffold protein mDia, which in turn promotes assembling of Apc and EB1. This complex stabilizes microtubules (Wen et al, 2004).

Calcium influx and protein kinase C activation in response to Dvl activation by Wnt through Frizzled are features of the calcium/Wnt pathway. Downstream of this pathway is activation of calcium/calmodulin dependent protein kinase II (CaMKII). This event promotes translocation of NF-AT (nuclear factor of activated T-lymphocytes) to the nucleus. The precise role of this pathway is not known. Saneyoshi et al. (2002) showed in a *Xenopus* animal model that this pathway can work through suppression of the canonical Wnt pathway. Stathmin is a protein which causes microtubule disassembly. Activation of CaMKII promotes phosphorylation of Stathmin. Also Stathmin is phosphorylated by MAPK which is downstream of PKC. Stathmin phosphorylated by these proteins can no longer destroy microtubules. Therefore one of the effects of the Calcium/Wnt signaling pathway is stabilization of microtubules (Curmi et al, 1999).

There are cross interactions of Wnt pathways. Activation of CaMKII leads to phosphorylation of Tcf. Phosphorylated Tcf can not activate transcription of target

genes. Also β-catenin/Tcf complex activates transcription of Xnr3, which in turn activates CaMKII (Saneyoshi et al, 2002). Two kinases, Jnk (Zhu and McKeon, 2000) and Gsk3β (Jhun et al, 2005), can phosphorylate NF-AT thus promoting its nuclear export. PKC can phosphorylate Gsk3β, which leads to inhibition of Gsk3β activity (Chen et al, 2000). IQGAP binds either F-actin or Ca^{2+}/calmodulin but not both together. Complexes of F-actin with IQGAP inhibit assembling of IQGAP with Ca^{2+}/calmodulin into a complex, and vice versa (Briggs and Sacks, 2003).

As described above, all Wnt pathways include activation of Dvl, after which the pathways separate. There are three Dvls discovered in mammals: Dvl1, Dvl2, and Dvl3. All of them have three highly conserved domains: an N-terminus DIX domain (binds Dvl and Axin), a middle PDZ domain (binds Postsynaptic density-95, Disk Large, and Zonula occludens-1), and a C-terminus DEP domain (binds Dvl, Eng-10, Pleckstrin). DIX and PDZ domains are used in canonical Wnt/signaling, whereas non-canonical pathways utilize PDZ and DEP domains.

There are 19 *Wnt* genes and 10 Frizzled receptors identified in mouse (Nusse, Mouse Wnt homepage, 2007). It is not clear how Dvl distributes these signals to three different pathways. It is not clear also which Wnt ligand can interact with which Frizzled receptor, and which receptor binds which ligand. Some of the Wnts are crucial for development of the nervous system. Wnt1 knockouts show defects in neural crest derivatives and lose cerebellum and the midbrain (McMahon et al., 1992) *Wnt3a* mutants have a deficiency of neural crest derivatives and lose the hippocampus, as was shown in experiments with *Wnt3* and *Wnt1* null mice by Ikeya et al. 1997. *Wnt3a* null

mice show that Wnt3a has a role in development of the hippocampus because mutant animals show under-proliferation of normally specified progenitors in the caudal-medial region of the dorsal telencephalon (Lee et al., 2000).At mid-gestation the hippocampus was absent or drastically reduced (Lee et al., 2000). Cell culture experiment on neural stem cells demonstrated that Wnt3a promoted their differentiation into astrocyte and neural lineages (Muroyama et al, 2004). Wnt4 seems to be involved in axon guidance of commissural axons. Lyuksyutova et al (2003) showed in the rat spinal cord explant culture that growth cones of post-crossing commissural axons are enlarged in the presence of Wnt4. Experiments of Hirabayashi and co-workers (2004) showed that overexpression of Wnt7a during cerebral cortex development promoted neuronal differentiation. *Wnt7a* mutants have a delay in maturation of glomerular rosettes and accumulation of synapsin I in the cerebellum, which suggests a role of Wnt7a in synaptogenesis. Canonical Wnts include Wnt1, Wnt3a, and Wnt8, whereas Wnt5a and Wnt11 are referred as non-canonical Wnts (reviewed by Cadigan and Liu , 2006). However this division for two subclasses is not very strict because some canonical molecules can activate non-canonical pathways. For example, Wnt1 and Wnt3a can activate PCP pathway in mammalian cell lines (Kishida et al, 2004). This activation could be inhibited by the Rho-binding domain of Rho-kinase (Kishida et al., 2004). The reverse example is activation of the canonical Wnt pathway by Wnt5a. Overexpression of *Wnt5a* in mammary epithelial cells led to induction of Cyclin D1 expression through the beta- catenin/Tcf route (Civenni et al, 2003).

1.11.8 Beta-catenin stabilization

The regulation of beta-catenin level is the key function of Apc in controlling tumourogenesis. It has been shown in tumor studies that *Apc* mutation leads to increased activation of beta-catenin/Tcf signaling in the cells, which may promote proliferation (Korinek et al., 1997, Morin et al., 1997). Also experiments with induction of expression of full-length Apc in cells which lack Apc showed that beta-catenin/Tcf/Lef signalling is reduced, and proliferation rate is decreased (Faux et al., 2004). However as was shown in that human colorectal carcinomas can develop without *APC* mutation when beta-catenin is activated by exon 3 deletion (Iwao et al., 1998), which removes sites indispensable for its phosphorylation.

Stabilization of beta-catenin is used to investigate consequences of constitutively active Wnt signalling pathway. It was shown that stabilization of beta-catenin in intestinal tissue can promote formation of tumors (Harada et al., 1999). Chenn and Walsh (2002) found that stabilization of beta-catenin in neural precursors causes enlargement of the surface area of the cerebral cortex. The brains of transgenic mice do not develop tumors or morphologic alterations if expressing stabilized beta-catenin in differentiated neurons, showing that postnatal Wnt signaling in differentiated neurons may not be sufficient to induce CNS tumourogenesis (Kratz et al., 2002)..

1.12 *Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$ as a model to understand roles of Apc in the developing cerebral cortex.

Apc is widely expressed in different tissues (Midgley et al., 1997). A high level of Apc expression is found during central nervous system development (Bhat et al,. 1994). As was shown in previous sections, Apc has many functions and is involved in many signaling pathways, which are important for development. However its role in central nervous system development is not well understood.

The main aim of my thesis is to establish what roles Apc plays in development of the telencephalon. Since *Apc* $^{-/-}$ animal die at gastrulation, I used the Cre-loxP system (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) to inactivate Apc specifically in the developing dorsal telencephalon.

The broad range of functions described for Apc suggests a number of possible functions during development. Connections between Apc and the canonical Wnt pathway lead to a hypothesis that the deletion of Apc will over-activate this pathway through stabilization of beta-catenin. Stabilization and nuclear translocation of beta-catenin can be visualized by immunostaining with anti-beta-catenin antibody. Besides beta-catenin stabilization and/or nuclear translocation it is important to know if this stabilization mimics activation of the canonical Wnt signaling. Up-regulation of expression of Wnt target genes, such as C-myc or Cyclin D1, or increased production of beta-galactosidase in Bat-Gal reporter embryos will provide evidence for Wnt signaling activation. An increase in Wnt inhibitor *Axin2* expression will provided additional evidence of the canonical Wnt activation.

Multiple important interactions of Apc in the cytoskeleton suggest that *Apc* mutation will interfere with its normal functioning. Possible adhesion, migration, and neurite outgrowth effects of the mutation can be tested to reveal abnormalities in these processes. Adhesion can be tested by visualizing changes in distribution and quantity of intercellular tight junctions and/or by cell culture experiments with different adhesion substrates. Migration during the cell cycle or maturation can be tested by visualizing particular populations of cells in the cortex and comparing their localization to the control ones. Cytoskeletal markers will show if there are defects in formation of neurites.

Because Apc mutations were found in many cancers there is a suggestion that cell cycle and apoptosis regulation are compromised in the developing dorsal telencephalon. Investigation of cell cycle and S-phase length, sizes of populations in different cycle phases, and apoptosis will clarify the effect of the *Apc* mutation on the dorsal telencephalon. Cell DNA content can be tested to find if the *Apc* mutation promotes ploidy defects, as a result of a disruption of Apc functions in chromosome segregation and mitotic spindle assembling

Deregulation of Wnt signaling might have effect on patterning and specification of the cerebral cortex. An analysis with markers of different brain areas and tissues will reveal changes of the patterning and specification in the dorsal telencephalon.

Thus, this work will provide detailed but preliminary analysis of major Apc roles in development of the dorsal telencephalon. However it is not possible to test every aspect of Apc functioning in the cortex, therefore there will be gaps to fill in for a future researcher.

2 Material and Methods

2.1 Animals

All animals were housed and cared for under Home Office regulations.

2.1.1 Mouse lines

2.1.1.1 ApcLoxP colony

Apc$^{580S/580S}$ animals are viable. Colony was maintained as homozygotes by breeding male and female *Apc*$^{580S/580S}$ animals. Mice have CBA/C57Bl6 mixed background

2.1.1.2 Rosa26 colony

Apc$^{580S/580S}$*Rosa26*$^{+/+}$ animals are viable. Colony was maintained as homozygotes by breeding male and female *Apc*$^{580S/580S}$*Rosa26*$^{+/+}$ animals. Mice have CBA/C57Bl6 mixed background.

2.1.1.3 APBAT colony

Apc$^{580S/580S}$*BatGal*$^{+/+}$ or *Apc*$^{580S/580S}$*BatGal*$^{+/-}$ animals are viable. Colony was maintained by breeding male and female *Apc*$^{580S/580S}$*BatGal*$^{+/+}$ and/or *Apc*$^{580S/580S}$*BatGal*$^{+/-}$ animals. Mice have CBA/C57Bl6 mixed background.

2.1.1.4 Emx1Cre colony

$Emx1^{Cre/Cre}Apc^{580S/+}$ or $Emx1^{Cre/+e}Apc^{580S/+}$ animals are viable. Colony was maintained by breeding male and female $Emx1^{Cre/Cre}Apc^{580S/+}$ and/or $Emx1^{Cre/+e}Apc^{580S/+}$ animals. Mice have CBA/C57Bl6 mixed background.

2.1.1.5 Mutant generation.

Females from ApcLoxP, or Rosa26, or APBAT colonies were plugged by males from Emx1Cre colony with $Emx1^{Cre/Cre}Apc^{580S/+}$ or $Emx1^{Cre/+e}Apc^{580S/+}$ genotypes. A plug day was designated as E0.5. Females were killed by neck dislocation on required embryonic day. Then embryos were dissected from uteri and either fixed or dissected further for analysis of primary cell cultures.

2.1.2 Injection of S-phase tracers

For continuous bromodeoxyuridine (BrdU) labeling pregnant females were injected intra-peritoneally with 200µl of 100µg/ml (in 0.9% NaCl)(Sigma) every two hours for six injections in total. Embryos were collected after 12 hours from the fist injection. Double labeling experiments were done by intra-peritoneal injection of iododeoxyuridine (IddU) (200µl of 100µg/ml in 0.9% NaCl)(Sigma) and BrdU 1.5 hour later. Animals were sacrificed after 0.5 hour of BrdU or 2 hour after IddU injection.

2.2 Mouse Genomic DNA Extraction and Genotyping

2.2.1 Phenol extraction

Mouse tail ear clips (1 to 4 clips) or embryo hind quarters (1) were digested overnight in 500µl tail tip lysis buffer (TTLB) containing 100µg/ml Proteinase K (Sigma). Digests were mixed with 500µl PCIA (phenol: chloroform: isoamyl alcohol 50:49:1) for 20 minutes and centrifuge at 13K for 5 minutes. The upper aqueous layer was removed to a fresh tube (optional step: 300µl PCIA was added and mixed then rotated for 15 minutes and .centrifuged at 13K for 5 minutes. 200µl of the upper aqueous layer was removed to a fresh tube). 1/10 volumes of sodium acetate pH5.5 and 2 volumes of 96% ethanol were added to the recovered aqueous portion and mixed until DNA strands visible before centrifuging at 13K for 5 minutes and removing the supernatant. Pellet was washed with 500µl 70% ethanol, spun at 13K for 1 minute and supernatant removed. Pellet dried for about 30 minutes at 37°C and then dissolved in 100µl ddH_20 with shaking overnight at 37°C.

2µl of DNA was usedfor subsequent PCR reactions.

Tail tip lysis buffer (TTLB)

200mM Tris pH8, 200mM NaCl, 5mM EDTA, 0.2% SDS

(Make up without SDS, autoclave, then add SDS)

2.2.2 Polymerase Chain Reaction (PCR) genotyping primers

Allele	Forward 5' Primer	Reverse 3' Primer	Product Size (bp)
Apc	5'-CACTCAAAACGCTTTT GAGGGTTGAAT-3'	5'-GTTCTGTATCATGGAAAGATAGGT GGT-3'	226 (wt) 314 (580)S)
Emx1	5'-TGGCCCAACTCGGTGTTAGG-3'	5'-CCACCAAGGACTCTATGGTG-3'	260
CRE	5'-ACCTGATGGACATGTTCAGGGATC-3'	5'-TCCGGTTATTCAACTTGCACCATG-3'	108
Rosa26	5'-AAAGTCGCTCTGAGTTGTTAT-3'	ROSA26)5'-GCGAAGAGTTTGTCCTCAACC-3' WT) 5'-GGAGCGGGAGAAATGGATATG-3'	1300 (ROSA) 550(WT)
LacZ	5'-CGAAATCCCGAATCTCTATCGTGC-3'	5'-GATCATCGGTCAGACGATTCATTG G-3'	400

2.2.3 Cycling conditions for PCR genotyping

Cycling conditions were as follows: -

For Apc primers:

94°C for 5 minutes

[94°C for 30 seconds, 59°C for 30 seconds and 72°C for 40 seconds] for 35 cycles.

For Emx1 and Cre primers:

94°C for 2 minutes

[94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds] for 35 cycles.

72°C for 7 minutes

4°C

For Rosa26 primers:

94°C for 1 minute and 30 seconds

[94°C for 20 seconds, 67°C for 45 seconds and 72°C for 1 minute] for 35 cycles.

72°C for 2 minutes

10°C

For LacZ primers

95°C for 5 minutes

[94°C for 1 minute, 64°C for 1 minute and 72°C for 2 minutes] for 35 cycles.

72°C for 10 minutes

2.2.4 PCR Reaction Mix

The following reaction mix was used for detection of all genotyping (see above). Prepare 19µl mastermix per sample and prepare enough for n+1 samples.

ddH2O	12.7
10mM dNTP's	0.4
5x PCR Buffer (Promega)	4
25mM MgCl2	1.2
PrimerFor	0.25
PrimerRev	0.25
Taq (Promega)	0.2

2.2.5 Agarose Gel Electrophoresis.

2% agarose(Sigma) solution was prepared by adding 1g of agarose into 50ml 1X TBE. The mixture was microwaved on full power for 2 minutes, evaporation losses were replaced with ddH_20. 1µl ethidium bromide (10µg/ml) was added (Fisher Scientific) and mixed gently. Agarose was poured into a gel casting tray, a comb was immediately inserted Agarose was allowed to set for 30 minutes. DNA sample was prepare by adding 5µl 5X loading buffer per 20µl PCR reaction 10µl of sample was loaded per well. 10µl (0.5µg) of DNA molecular weight marker XIV (Roche) was loaded in one well. Gels were run at 80V for ~ 30 minutes, or until bands have resolved. Visualization was done by exposure to UV (ultraviolet) light

Molecular weight ladders: -

DNA molecular weight marker

Working dilutions of DNA molecular weight marker:

1µl DNA molecular weight marker

5µl 5X gel loading buffer

19µl ddH_20

2.3 Histology.

2.3.1 Fixation of Brain Tissue.

Embryonic heads, or whole embryos (E12.5 and earlier) were submerged in a solution of 4% paraformaldehyde in 1x PBS for 8 - 16 hours at 4°C on a rocking platform. The heads were then washed with 1xPBS and either (1) dehydrated through a series of increasingly-concentrated ethanol solutions prior to embedding in paraffin wax, carried out using an automated tissue processor (Tissue-Tek VIP, Sakura) or (2) in the case of frozen sections, heads were placed in a 30% sucrose:PBS solution for cryoprotection and left overnight at 4°C with agitation until the heads sank. Heads were placed in OCT (Lamb), positioned in the horizontal, coronal or sagittal plane, frozen on dry ice and then stored at -80°C.

2.3.2 Microtome Sectioning of Wax Sections.

Wax-embedded heads were cut into 10μm-thick sections on a microtome (Reichert-Jung 2050) and sections floated onto poly-l-lysine (Sigma) slides. Sections were left to dry at 37°C overnight before immunohistochemistry or haematoxylin and eosin staining.

2.3.3 Cryostat Sectioning.

Heads for cryostat sectioning were removed from storage at -80°C and equilibriated at -20°C to -26°C for one hour in the cryostat. 10μm-thick sections were cut on the cryostat and collected onto Superfrost Plus (BDH) slides. All sections were air-dried prior to storage at -80°C with a dessicant until use. Prior to immunohistochemistry, slides were thawed thoroughly at room temperature in their slide box.

2.4 Immunohistochemistry.

2.4.1 Chromogenic endpoint

Sections were dewaxed in xylene and rehydrated through a series of solutions of descending ethanol concentration to water. Endogenous peroxidise activity was eliminated through incubation in 3% hydrogen peroxide solution with 10% methanol for 5 minutes. DNA was denatured and antigens unmasked by heat treatment in 10mM

sodium citrate (pH6) in a microwave oven. Evaporation losses were replaced and slides cooled for at least 20 minutes on ice prior to washing in 1xPBS. Blocking solutions contained 10% goat serum (NGS) in PBS with 0.1% Triton-X-100 (Sigma). Following incubation with the primary antibody (4°C overnight), slides were washed 3x with 1xPBS with 0.1 Triton-X-100 followed by incubation with species-specific HRP-conjugated secondary antibodies for 1 hour at room temperature using a Envision^{+} Kit (Dako). Following three washes in 1xPBS with 0.1 Triton-X-100, antibody staining was revealed using 3,3'-diaminobenzidine (DAB), which produces a brown precipitate. In some cases sections were counterstained with Harris-hematoxylin (Thermo Shandon). Slides were dehydrated through increasing concentrations of ethanol, cleared in two changes of xylene and then mounted under coverslips in DPX (BDH).

2.4.2 Fluorescent endpoint

For immunofluorescence section slides were processed identically to chromogenic reactions with a few alterations. If two primary antibodies were used simultaneously, blocking solutions contained 20% goat serum (NGS) in PBS with 0.1% Triton-X-100 (Sigma). After applying secondary antibodies and following washes dewaxing and rehydration steps did not need to be performed. Following incubation with the primary antibody, species-specific secondary antibodies conjugated to fluorescent molecules were used at 1:200 dilution: goat anti-mouse or goat anti-rabbit Alexafluor-488 or Alexafluor-568 (Molecular Probes). For double immunohistochemistry to detect two different primary antibodies, primary antibodies from two different species were

chosen to avoid cross-reactivity and incubated together. This was followed by detection using Alexafluor-488 and -568 secondary antibodies. For detection of IddU/BrdU mouse anti-IddU/BrdU antibodies were used (clone B44, 1:100 in blocking solution; Becton Dickenson). Rat anti-BrdU antibodies (clone BU1/75, 1:100; Abcam) were used to detect BrdU but not IddU. In case of double labeling goat anti-mouse highly cross-absorbed antibodies were used to prevent cross-reactivity. Nuclei were counterstained using the DNA dye TO-PRO-3 iodide (Molecular Probes) at 1:100 dilution in water. Sections were mounted under coverslips using Mowiol (see below) to prevent fading of fluorescence.

2.4.2.1 Preparation of Mowiol Mounting Medium

2.4g Mowiol (Calbiochem Cat no 475904) were added to 6g of Glycerol (Glycerol density =1.26g/ml – for 6g = 6/1.26 = 4.76mls) (in a 50ml conical flask) and then stirred with a pipette to mix. 12ml dH_20 were added and left stirring for several hours or overnight at room temperature. 12ml 0.2M Tris (pH8.5) (1M Tris = 121.14 g/l, 100nM = 12.114g/l 200nM = 24.228g/l or 2.423g/100ml) were added and heated to 50°C for 1 – 2 hr or until Mowiol was completely dissolved, then centrifuged at 2000rpm for 15 minutes. 1,4-diazobicyclooctane (DABCO) Sigma(Antifade) to 2.5% (=0.72g) was added and aliquoted. Aliquots were stored at -20°C. Aliquots were centrifuged before use to pull down any bubbles. After prolong storage aliquots were warmed at 50°C for 5 minutes to make solution uniform.

Mowiol mounting medium solidifies once used and allows direct mounting of the coverslips onto glass slides. Once mounted, the coverslips can be viewed with immersion lenses as the coverslips will not move. The coverslips are also removeable by gently sliding off the side of the slide, using tweezers. The Mowiol can be removed by immersing the coverslips in ddH_20 for a few minutes.

2.4.3 Antibodies used

Primary antybodies	Species	Clone name	Monoclonal/ polyclonal	dilution	Source
Apc(C-20)	rabbit	sc-896	polyclonal	1/400	Santa Cruz
Beta-catenin	mouse	110154	monoclonal	1/200	Transduction Laboratories
Beta-III-tubulin	mouse	Tuj1	monoclonal	1/800	Sigma
PCNA	mouse	PC10	monoclonal	1/400	Dako
BrdU	rat	BU1/75	monoclonal	1/100	Abcam
BrdU/IddU	mouse	B44	monoclonal	1/100	BD Biosciences
Calretinin	rabbit	AB5054	polyclonal	1/400	Chemicon
Nestin	mouse	RAT401	monoclonal	1/50	DSHB
Nkx2.1	mouse	8G7G3/1	monoclonal	1/100	Abcam
Pax6	mouse	Pax6	monoclonal	1/200	DSHB
Olig2	rabbit	Ligon et al., 2004	polyclonal	1/10000	D.Rowitch
Mash1	mouse	24B7.2d11	monoclonal	1/100	BD Pharmigen
Islet1	mouse	40.2D6	monoclonal	1/100	DSHB

Pax3	mouse	Pax3	monoclonal	1/400	DSHB
Ngn2	mouse	7G4	monoclonal	1/10	D.Anderson
Tbr1	rabbit	Englund et al 2005	polyclonal	1/500	R.Hevner
Tbr2	rabbit	Englund et al 2005	polyclonal	1/500	R.Hevner
Foxg1	rabbit	Watanabe et al., 2005	polyclonal	1/500	Y.Sasai
P21	rabbit	SX118	polyclonal	1/100	BD pharmigen
C-myc	mouse	9E10	monoclonal	1/200	Roche
Cyclin D1	rabbit	Ab31450	polyclonal	1/400	abcam
N-cadherin	mouse	610920	monoclonal	1/200	BD Bioscience
Beta-galactosidase	mouse	Ab7981	monoclonal	1/800	abcam
Pericentrin	rabbit	PRB-432C	polyclonal	1/400	Covance
Phosphohistone -3	rabbit	H9908	polyclonal	1/400	Sigma
WT1	mouse	6F-H2	monoclonal	1/1000	Dako
CK PAN	mouse	C-2562	monoclonal	1/10,000	Sigma
VIM	mouse	3B4	monoclonal	1/200	Dako
SMA	mouse	1A4	monoclonal	1/50	Dako
MYO D1	mouse	5.8A	monoclonal	1/100	Dako
Desmin	mouse	D33	monoclonal	1/100	Dako
S100	rabbit	Z0311	polyclonal	1/1000	Dako
Secondary antibodies	Species		Fluorochrome	dilution	Source

Anti-mouse	goat		Alexa488	1/200	Molecular probes
Anti-rat	goat		Alexa568	1/200	Molecular probes
Anti-rabbit	goat		Alexa568	1/200	Molecular probes

DSHB stands for Developmental Studies Hybridoma Bank. CK PAN clones: C-11, PCK-26, CY90, KS-1A3, M20, A53-B/A2

2.4.4 Detection of apoptosis by TUNEL

Terminal deoxynucleotidyl nick end labeling (TUNEL) was performed according supplier protocol (Roche). TUNEL labeled sections were visualized at 488 nm fluorescence.

2.5 Staining for Bacterial LacZ (β-galactosidase)

2.5.1. Reagents.

LacZ Fixative (volumes for making 20ml)

4% paraformaldehyde in PBS	20ml
0.02% NP40	40µl of 10% stock
0.01% sodium deoxycholate	20µl of 10% stock
5mM EGTA	200µl of 0.5M stock
2mM $MgCl_2$	40µl of 1M stock

LacZ Wash (volumes for 100ml)

PBS	100ml
2mM $MgCl_2$	200µl of 1M stock
0.02% NP40	200µl of 10% stock
0.01% sodium deoxycholate	100µl of 10% stock

LacZ staining buffer (volumes for 10 ml)

PBS	10ml
2mM MgCl2	20µl of 1M stock
0.02% NP40	20µl of 10% stock
0.01% sodium deoxycholate	10µl of 10% stock
5mM Potassium ferricyanide	100µl of 0.5M stock
5mM Potassium ferrocyanide	100µl of 0.5M stock
1mg/ml X-gal	250µl of 40mg/ml stock in dimethylformamide (DMF) (kept in freezer).

LacZ stop solution (20mM EDTA in PBS)

20ml PBS + 800µl 0.5M EDTA

LacZ post-fix (2% Glutaraldehyde in PBS).

20ml PBS + 800µl 50% Glutaraldehyde.

2.5.2. Protocol

Embryos were dissected in ice cold PBS and fixed with shaking at 4°C for 1 hour in LacZ Fixative. Fixed embryos were washed 3 x 20 minutes (minimum) at room temperature shaking in LacZ Wash and stained in X-Gal at 37°C with shaking 1 hr to overnight (or longer) in the dark. Staining was stopped by wash in LacZ Stop Solution. Then tissue was postfixed in LacZ fix solution. For cutting sections embryos were transferred to 50% ethanol and proceeded with wax embedding [X-gal product leaches in organic solvents but persists]. For cutting cryostat sections appropriate protocols were used.

In some cases with appropriate fixation for cryostat done embryos were cut and then staining. This applies to cryostat sectioning only

Cryostat sections can be counterstained with Nuclear Fast Red (Fisher).

2.6 Primary Cell Culture and FACS analysis

The telencephalon was isolated and dissected in ice-cold oxygenated Earle's buffered salts solution (EBSS). The dorsal telencephalon was separated from the ventral telencephalon. Telencephalic tissue was dissociated using Papain dissociation system kit (Worthington Biochemical Corporation) according to the supplier's protocol. Then cells were resuspended in serum-free medium. Concentration of cells were counted using hemocytometer. After this step cells could be used for different experiments.

1) Cell from the control and the mutant were plated at the similar density about 1500 cell/mm^2. Cells were plated on glass 9mm in diameter cover-slips (BDH) coated with poly-lysine (0.01% for 30 minutes), laminin (1mg/ml for 30 minutes), fibronectin

(1mkg/ml for 30 minutes), or gelatin (0.1% for 30 minutes). 4 hours plating cells were fixed in 2%PFA for 10 minutes, then washed with 1xPBS 0.2% Triton-X-100 for 10 minutes. TO-PRO-3 was applied at 1/2000 dilution for 3 minutes. After wash with double-distilled water for 3 minutes coversliops were mounted with Mowiol mounting media on frost-glass (BHD). Cells were visualized in Leica fluorescent microscope.

2) Collagen embedded cultures on cover-slips were used to prevent fall off of the mutant cells. Collagen preparation was flatten on 9 mm in diameter cover-slip (BHD) and settle for 20 minutes at 37°C. Then cell in liquid collagen were added on top and left to set for 20minutes at 37°C. Cell from the control and the mutant were plated at the similar density about 1500 cell/mm^2. After collagen was set 0.5ml of serum-free medium was added on top. Cultures were incubated for 4 hours at 37°C. Then cultures were fixed in 2% PFA for 20 minutes and washed in 1xPBS 0.2%Triton-X-100 for 20 minutes. Blocking was performed in 10% NGS in 1xPBS 0.1% Triton-X-100 for 30 minutes. Primary Apc (1/500) antibody was applied for 1 hour at room temperature. Wash was performed with 1xPBS 0.1% Triton-X-100 for 30 minutes. Then appropriate secondary antibody was applied (1/200) in block solution for 45 minutes at room temperature. Wash was repeated. Nuclei were counterstained with TO-PRO-3 (1/2000) for 3 minutes with following wash in double-distilled water for 5 minutes. Then cover-slips were mounted with Mowiol to a frost glass (BHD).

3) Cell used for FACS analysis were fixed in -20°C 70% ethanol for ≥2 hours on ice. Then centrifuge for 5 minutes at 200g and ethanol discarded. Cells were resuspended in PBS for 1 min and centrifuged again for 5 minutes at 200g. Cell pellet was thoroughly resuspended in PBS containing 0.05mg/ml of Propidium Iodide (Sigma)

and 0.5 mg/ml of RNAseA (Roche). FACS analysis was performed in the same or next day on FACSCalibur (BD Biosciences) which runs CellQuest software.

2.6.1. Collagen mixture for embedded cultures.

Prepared by mixing the following reagents together on ice under sterile conditions. This mixture was made up fresh for each culture experiment.

180µl rat tail collagen type 1 (BD Biosciences, #354236)

180µl bovine collagen type 1 (BD Biosciences, #354231)

40µl 10X DMEM (Sigma, #D7777) - reconstitute powder using sterile ddH_20 and filter.

~ 100µl 0.8M sodium bicarbonate or enough to turn the mixture a pale pink colour

2.6.2. Earle's balanced salt solution ('EBSS')

Prepared by mixing the following reagents together under sterile conditions and stored for not more than two weeks at 4°C until use.

100 ml Earle's balanced salt solution 10X (Sigma, #E-7510)

0.22 g Na_2HCO_3 (Sigma, #S5761), final concentration 22 mg/ml

0.065 g glucose (Sigma, #G-7021), final concentration 6.5 mg/ml

900 ml double deionised water.

EBSS was oxygenated by bubbling with 95% O_2 and chilled on ice prior to use.

2.6.3 Serum-free culture medium

Prepared by mixing the following reagents together under sterile conditions and stored for not more than two weeks at 4°C until use. Medium was warmed and equilibriated in a 37°C humidified incubator containing 5% CO_2 for at least one hour prior to use.

100 ml F12, Hams (Sigma, #N4888)

100 ml Dulbecco's modified Eagles' medium (DMEM) (Sigma, #D5671)

1 mg insulin (Sigma, #I6634), final concentration 5μg/ml

2 mg apo-transferrin (Sigma, #T1147), final concentration 10μg/ml

3 ml HEPES buffer (Sigma, #H0887)

0.24 g Na_2HCO_3 (Sigma, #S5761), final concentration 0.12mg/ml

3 ml antibiotics stock: 100 mg gentamycin (Sigma, #G1264) and 200 mg kanamycin (Sigma, #K1377) added to 20 ml sterile double deionised water, filter-sterilised and stored at -20°C.

2 ml putrescene stock: 100 μM stock, 161.1mg/100ml in sterile double deionised water, filter-sterilised and stored at -70°C (Sigma, #P5780), final concentration 16.11μg/ml

20μl progesterone stock: 20 μM stock, 6.29mg/100ml ethanol stored at -70°C (Sigma, #P8783), final concentration 6.29ng/ml

20μl Na_2SeO_3 stock: 30 μM stock, 5.2mg/100ml in sterile double deionised water, filter-sterilised and stored at -70°C (Sigma, #S5261), final concentration 5.2ng/ml

2 ml L-glutamine stock: 0.2M stock, 6.344g/100ml in sterile double deionised water, filter-sterilised and stored at -70°C (Sigma, #G2128), final concentration 25μg/ml.

2.7 Microscopy

2.7.1 Light Microscopy.

Slides were photographed using a Leica DMLB upright compound microscope connected to a Leica DSC480 digital camera, using Leica IM50 image management software.

2.7.2 Confocal Microscopy

Fluorescent staining was viewed using a Leica DMRE compound microscope, part of a Leica TCS NT confocal system using 'Leica Lite' software. Alexafluor-488 staining was collected in the FITC (green) channel, Alexafluor-568 in the TRITC (red) channel and TO-PRO-3 in the Cy3 (far-red, pseudo-coloured blue) channel.

2.7.3 Enumeration of BrdU and IddU positive cells for cell cycle kinetic analysis at E12.5 and E13.5

Sections of rostral, middle, and caudal dorsal telencephalon were stained and imaged both medially and laterally with confocal microscope. Red, green and blue

channels were acquired for all images. On each image a box of 100μm length was counted laterally and medially. Three control and three mutant embryos were counted for each age. Cell cylkce parameters were calculated as described by Martynoga et al. (2005).

2.7.4 Calculation of BrdU and PCNA labeling index in the dorsal telencephalon at E12.5 and E13.5

Boxes 100μm length were counted in the dorsal telencephalon dorsally and medially in rostral, middle, and caudal regions. Three control and three mutant embryos were counted for each age. Results are expressed as proportion of all dorsal telencephalic cells.

2.8 Image Analysis

Some images were modified with Adobe Photoshop 7 software. When the brightness or contrast or images was adjusted to enhance detail, the changes were used to the whole image.

2.9 Statistical Analysis and Graph plotting

Sigmastat (Systat Software Inc.) was used for data analysis. Excel (Microsoft) was used to plot data.

2.10 Quantification of gene expression

2.10.1 RNA extraction

RNA was expected from the dorsal telencephalic tissue at E12.5, E13.5, and E15.5 using RNEasy mini kit (Qiagen) according to a protocol supplied by the producer and using DNAse (Roche) on the designated step. RNA was stored at -80.

2.10.2 cDNA synthesis

SuperScript III First-Strand Synthesis SuperMix (Invitrogen) was used to synthesize cDNA from RNA samples. The procedure was performed according to supplier's protocol using random hexameres.

2.10.3 Quantitative RT-PCR

Quantitative RT-PCR was performed using Quanti Tect SYBR Green PCR Kit (Qiagen) on Opticon single wavelength PCR machine (MJ Research) driven by Opticon Software. All primers were designed to span introns and have annealing temperature around 58°C. Lengths of products of all quantified genes were less than 250 base pairs to ensure linearity of SYBR Green incorporation.

Reaction was set up as specified by manufacturer. Opticon software was used to extrapolate transcript levels from PCR reaction kinetics. All transcript levels were normalized to *GAPDH* levels.

Number of samples was 3 (embryos) of the control and mutant at every developmental stage, except mutant at E12.5 were 5 samples were used.

2.10.3.1 The standard reagent mix for 1 PCR reaction

SYBR Green MasterMix 12.5µl

Primers 2x 1µl

Double distilled water 10.5 µl

2.10.3.2 The PCR program for all genes

95°C for 15 minutes

{94°C for 15 seconds, 55°C for 30 seconds, 72°C for 30 seconds, plate read taken} 35 cycles

Melting curve was performed from 60°C to 90°C with 1°C step.

2.10.3.3 Primers used for quantitative RT-PCR

	Forward primer	Reverse primer
Apc exon 4	5'-ccgttcaggagaatgcagtc-3'	5'-tgccgtcttgtcatgtctgt-3'
Apc exon 14	5'-gtgtccagcttgatagctac-3'	5'-caaggcttcctggtctttag-3'
Wnt1	5'-atacgaccccgtttctgctg-3'	5'-ttccactccctcacctcaaagc-3'
Wnt8b	5'-aaggcttacctggtctactc-3'	5'-cagagctgatggcgtgcaca-3'
Axin2	5'-acagtagcgtagatggagtc-3'	5'-ctgtggaacctgctgccttc-3'
p53	5'-AGAGACCGCCGTACAGAAGA-3'	5'-CTGTAGCATGGGCATCCTTT-3'

3. Apc deletion and its effect.

3.1 Introduction

The tumour suppressor protein Apc is widely expressed in the developing CNS (Bhat et al., 1994). As described in Chapter 1, Apc plays important roles in Wnt signalling. Wnt signalling is also important for CNS development (reviewed by Ciani and Salinas, 2005). We chose to examine the role played by Apc in cortical development by inactivating *Apc* specifically in the developing cortex using Cre-loxP gene technology.

We used the floxed *Apc* allele described by Shibata et al. (1997) – *580S*. In this allele, Cre activity leads to a deletion of *Apc* exon 14 and a frameshift at codon 580 (Fig 3.1, a, b). The Apc 580S mutation can have following effects on Wnt signalling: the canonical pathway is over-activated, because beta-catenin is no longer destroyed, and the planar cell polarity pathway is disrupted, as actin meshwork formation is inhibited. In this chapter, I examine the consequences of deletion of *Apc* in the developing cerebral cortex.

I used $Emx1^{Cre}$ to produce the mutation of *Apc* specifically in the developing dorsal telencephalon.

$Emx1^{Cre/+}Apc^{580S/+}$ embryos were used as controls for $Emx1^{Cre/+}Apc^{580S/580S}$ because there are reports of deleterious influences of Cre-recombinase during ontogenesis (Loonstra et al., 2001; Baba et al., 2005). Therefore differences in the mutant compared to control should be due to different number of Apc^{580S} copies in the

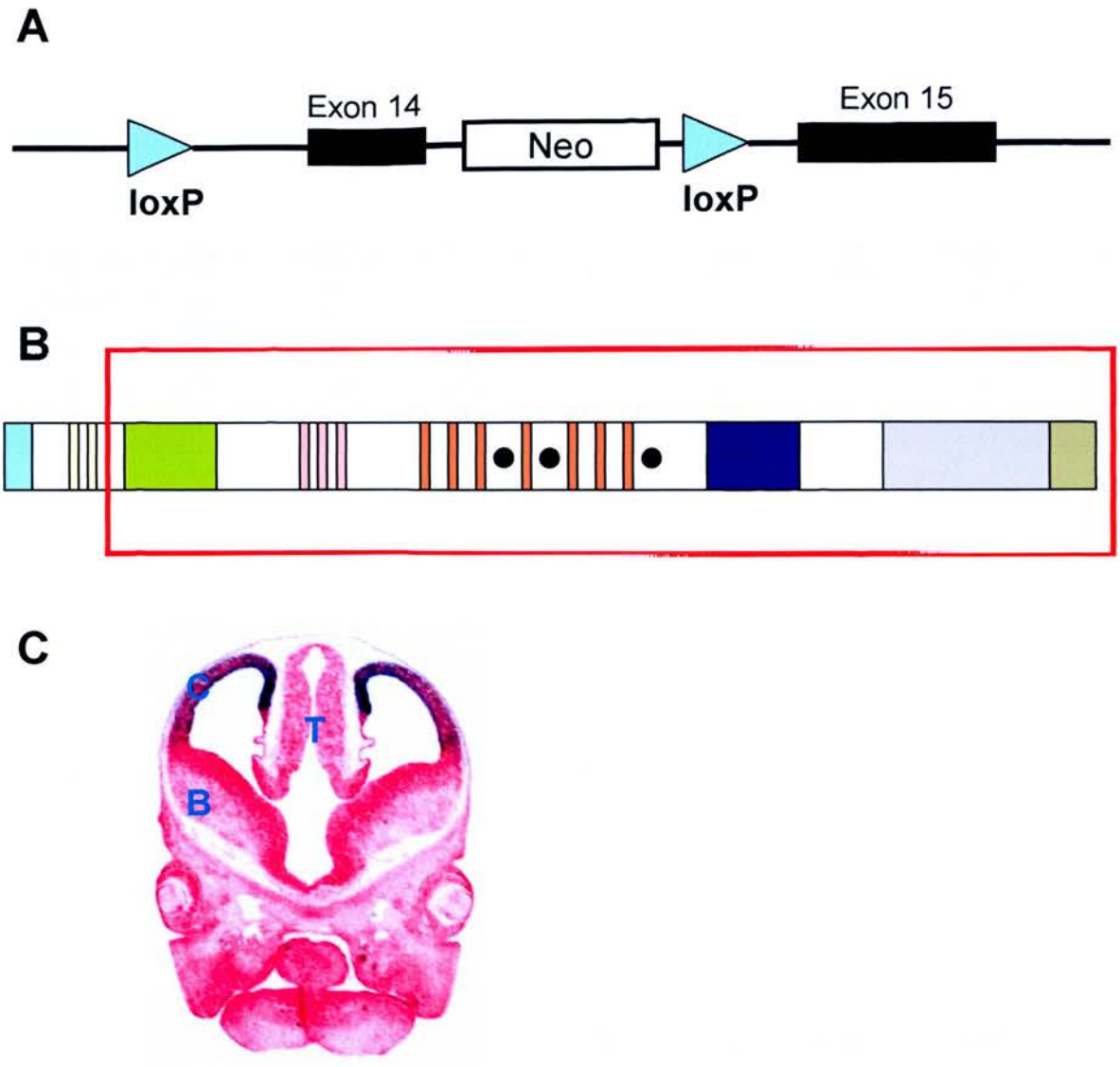


Fig 3.1. a) **Structure of *APC*^{580S} allele.** Cre-mediated deletion of exon 14 introduces a frameshift at residue 580 (modified from Shibata et al.,1997)
b) **Defect in Apc protein after the mutant.** Red box show areas which are affected by the 580S mutation (all domains are shown as on picture 1.7).
c) **Coronal section of ROSA26 reporter embryo at E12.5.** Blue staining is the region of the cortex where *Emx1*^{cre} is active. C – cortex, B – basal ganglia, T – thalamus. It is possible to see a high medial low rostral gradient of staining in the cortex. All other tissue looks negative.

embryo because Cre-recombinase is present in both the control and mutant. However no obvious differences were found in phenotype of $Emx1^{Cre/+}Apc^{580S/+}$ embryos compared to $Emx1^{+/+}Apc^{580S/+}$ embryos. Additionally usage of $Emx1^{Cre/+}Apc^{580S/+}$ or $Emx1^{Cre/Cre}Apc^{580S/+}$ studs did not reveal any differences from wild type animals in size, appearance, behaviour, or life span.

During experimental work $Emx1^{Cre/Cre}Apc^{580S/+}$ or $Emx1^{Cre/+e}Apc^{580S/+}$ studs were used to plug $Emx1^{+/+}Apc^{580S/580S}$ females. Collected embryos were genotyped and distributed into two groups according to genotype: control ($Emx1^{Cre/+}Apc^{580S/+}$) and mutant ($Emx1^{Cre/+}Apc^{580S/580S}$). Other genotypes were not used.

3.2 Progression of defect

Cre expression is driven by the *Emx1* promoter in the current model. *Emx1* is expressed from E9.5 of mouse development in the telencephalon as it starts to form (Yoshida et al., 1997). It is therefore logical to expect manifestation of the consequences of the mutation later than E9.5. To verify the region of Cre-activity, a *Rosa26* reporter allele (Zambrowicz et al., 1997) was used (Fig 3.1, c). Results in Fig 3.1 shows that Cre-recombination occurred in the cortex and that there is a gradient (high medial, low rostral) of staining intensity which might correspond to the gradient in *Emx1* expression.

Then I looked for morphological changes as a result of loss of *Apc* at a set of developmental stages after E9.5. The dorsal telencephalons (DTel) of the controls ($Emx1^{Cre/+}Apc^{580S/+}$) and the mutant ($Emx1^{Cre/+}Apc^{580S/580S}$) do not show any apparent

difference at E10.5 (compare Fig 3.2 a and b to Fig 3.2 c and d). Two days later the control DTel has a smooth structure (Fig 3.2 e and f) but the structure of the mutant DTel is affected: the ventricular surface is uneven (Fig 3.2 g and h), although the appearance of the hippocampus looks unaffected (Fig 3.2 h, black arrow). At E13.5 the control DTel tissue preserves its smooth structure (Fig 3.2 i and j). The developmental defect in the mutant DTel becomes more pronounced: unstructured conglomerates of cells are formed (Fig 3.2 k and l). At E15.5 it is possible to distinguish a layered structure in the control DTel (Fig 3.2 m and n). The mutant DTel is an unstructured cell mass (Fig 3.2 o and p).

3.3 Proof of deletion

An overview of the mutant suggests that the DTel is affected, which is expected because of using the *Emx1* promoter for Cre-recombinase expression. Immunostaining for Apc protein could reveal areas where the Apc protein is lost.

At E10.5 immunostaining for Apc does not show drastic differences in the control compared to the mutant (Fig 3.3. a, b and c, d, respectively). There is a possibility that Apc negative cells are not seen due to masking of single Apc negative cells by surrounding Apc positive ones. At E12.5 there is a difference in immunostaining of the control and mutant DTels. The control has Apc throughout the DTel (Fig 3.3 e and f). Regions which are negative for Apc staining can be found in the mutant DTel at the same age (Fig 3.3 g and h, black arrows). A very surprising staining result comes at E13.5. The control preserves Apc staining of the DTel (Fig 3.3 i and j) and the mutant

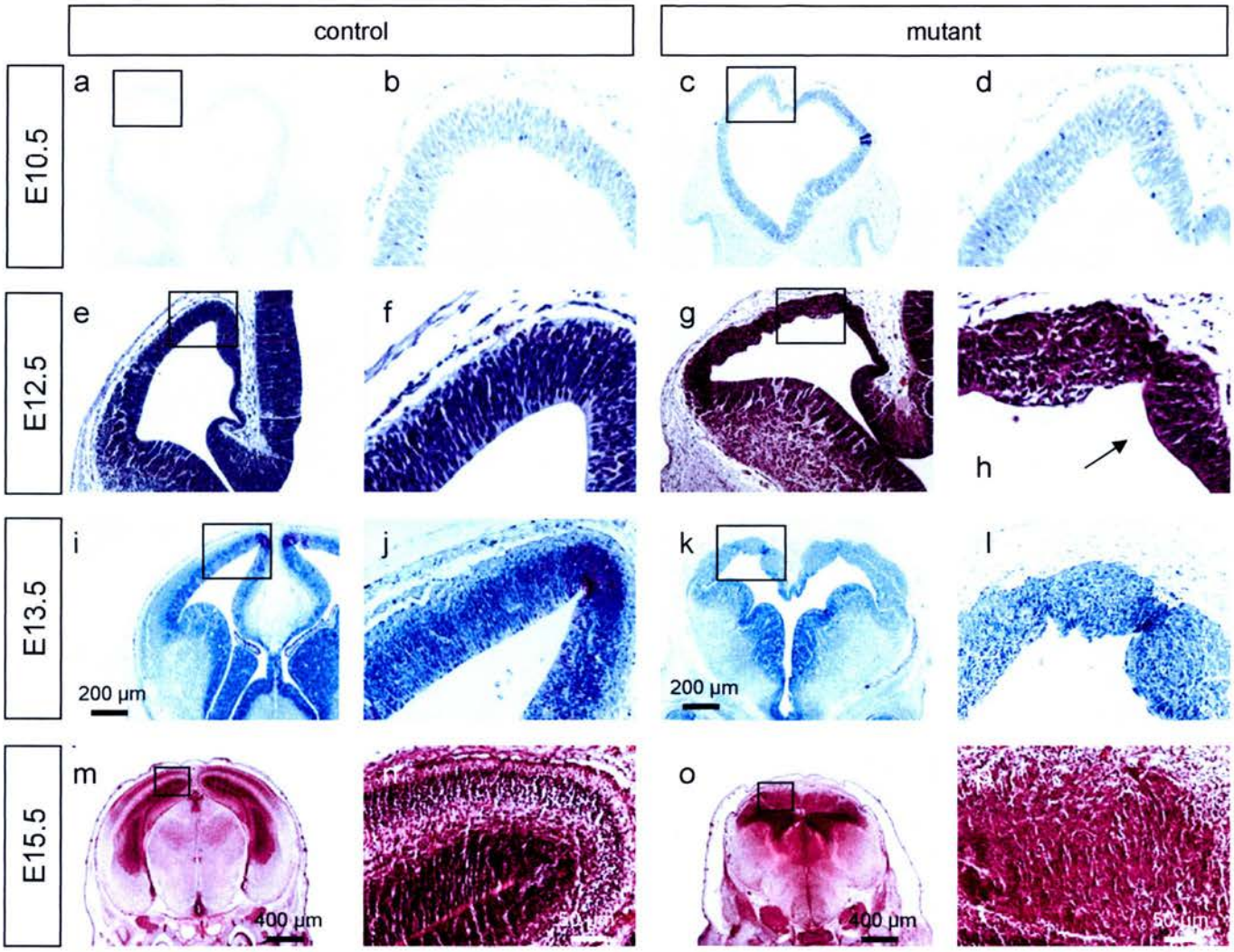


Fig 3.2 Progression of defects in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E10.5, E12.5, E13.5 and E15.5 (haematoxylin and eosin staining). At E10.5 the control (a and b) and the mutant (c and d) telencephalons are similar. At E12.5 the control DTel preserves a smooth structure (e and f) while the mutant DTel starts to crumple (g and h, black arrow shows the hippocampus). At E13.5 the control DTel develops normally (i and j), but the mutant DTel becomes more disorganized (k and l). At E15.5 the DTel of the control shows a normal smooth layered structure (m and n), whereas the mutant DTel presents a mass of tissue (o and p).

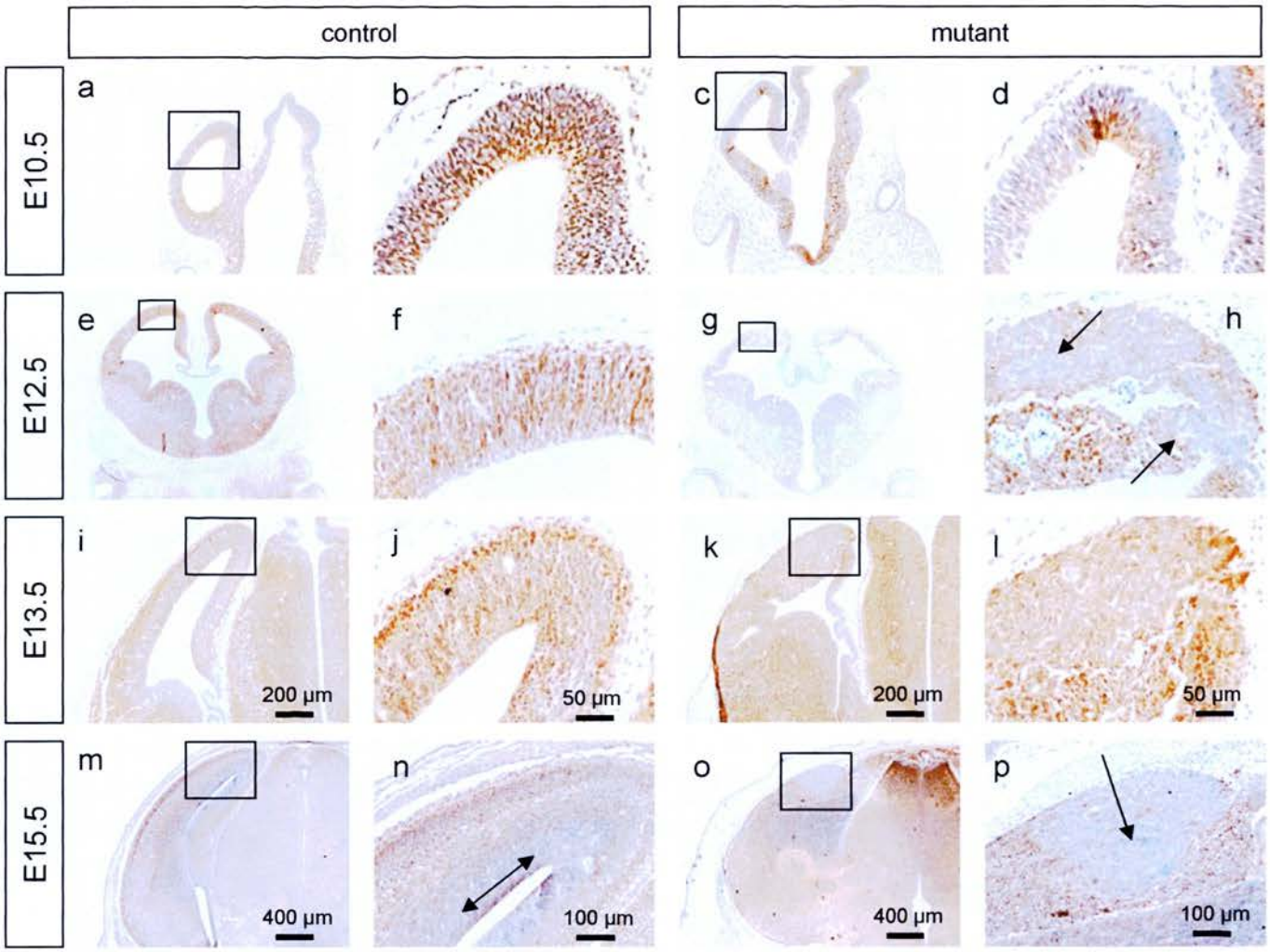


Fig 3.3 Immunostaining for Apc in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E10.5, E12.5. E13.5 and E15.5. At E10.5 Apc staining is similar in the control (a and b) and the mutant (c and d). At E12.5 there are patches of cells which are Apc negative (g, h, black arrows) while the control remains Apc positive (e, f). Surprisingly, at E13.5 both the control (i) and mutant (k) look positive for Apc. At 15.5 there is an obvious negative region in the mutant DTel (o, p, black arrow), while in the control DTel the ventricular zone looks slightly negative (m, n, double black arrow).

DTel is also immunopositive for Apc (3.3. k and l). Two days later the control DTel has lower levels of Apc staining in the VZ (3.3 m and n, black arrows). At the same age the mutant has an obvious negative region in the DTel (3.3 o and p, black arrow) surrounded by Apc positive tissue.

Other approaches to confirm *Apc* deletion were used.

First, a collagen embedded dissociated cell culture was performed on E13.5 DTel tissue followed by immunostaining for Apc. An embedded culture system was used because the mutant cells showed poor adhesion properties on different surfaces (section 3.4.5). Cells were cultured for 3 hours. After immunostaining and imaging the number of Apc positive and Apc negative cells was counted for the control and the mutant (Fig. 3.4 a and b, respectively). The percentage of Apc negative cells was determined and analyzed statistically. The results are presented in Fig 3.4 c. The data in Fig 3.4 c show that approximately 37% of the mutant cells are negative for Apc on average whereas the control has 9% of Apc negative cells on average. This difference is statistically significant ($p \leq 0.001$).

Secondly, quantitative RT-PCR was performed with DTel samples from the control and the mutant at E12.5, E13.5, and E15.5. Transcript levels of two exons were tested: transcript containing exon 4, which should be in both the control and the mutant, and transcript containing exon 14, which is deleted in the mutant. Exon 4's transcript expression profile shows a slight, significantly increased expression in the mutant DTel compared to the control at E12.5 (Fig 3.5). At E13.5 and E15.5 levels of expression of transcript containing exon 4 are similar in the control and the mutant samples (Fig 3.5). At E12.5 the mutant shows exon 14 transcript level similar to the control (Fig 3.6). Exon

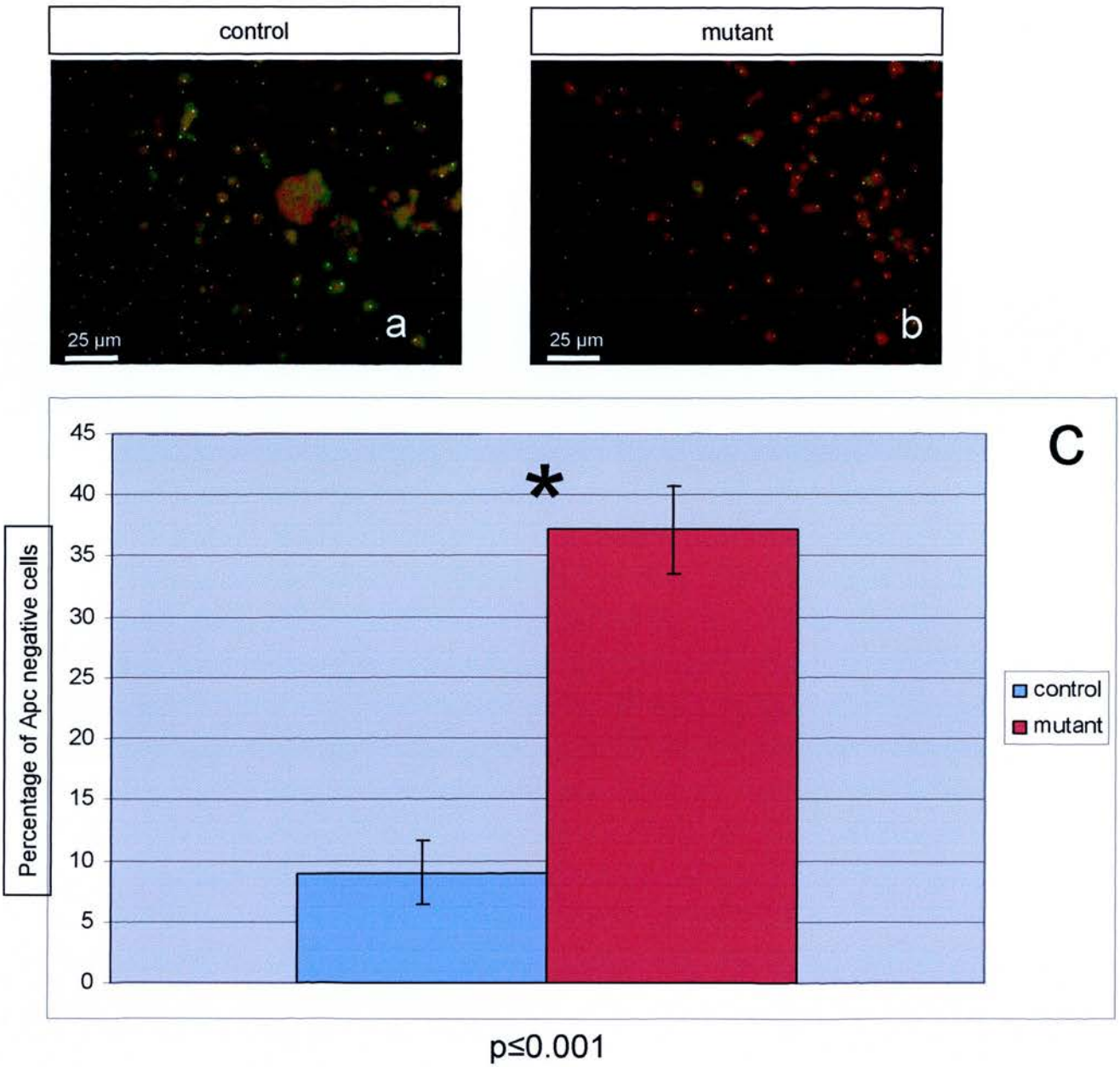
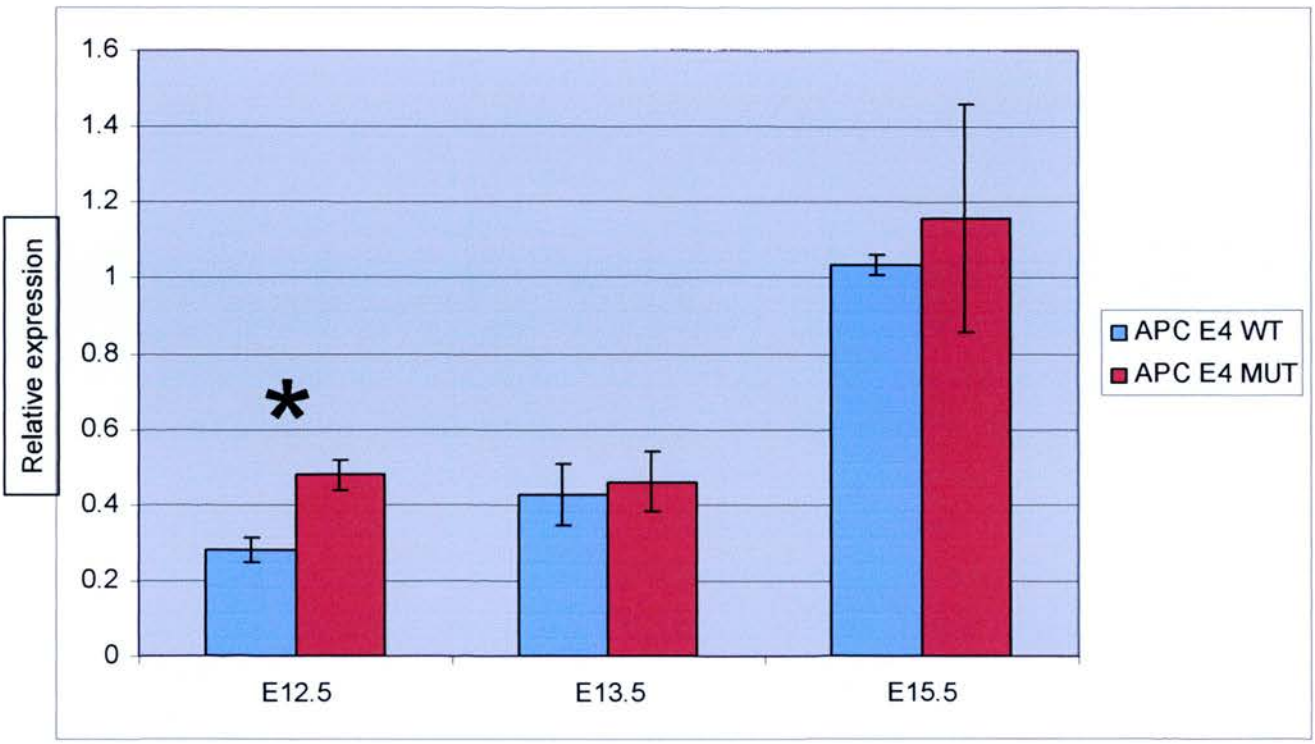


Fig 3.4 Immunostaining for Apc (green) and quantification of Apc negative cell number in a collagen embedded dissociated cell culture of DTel of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant *Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5. There is an obvious decrease in staining intensity in the mutant culture compared to the control (b and a, respectively). There is a significant increase in quantity of Apc negative cells in the mutant compared to the control (c). N= 4 and 5 embryos , t-test performed.*-significant difference. Error bars are SEM. White dots – Apc positive cells, yellow dots – Apc negative cells on a and b. Red colour – nuclear ToPro-3 staining.



p =0.015 p =0.777 p =0.7

Fig 3.5 Quantitative RT-PCR of *Apc* transcript containing exon 4 in the telencephalon of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5 and E15.5. There is up-regulation of *Apc* transcript containing exon 4 at E12.5. At E13.5 and E15.5 there is no significant difference between levels of transcript expression in the control and mutant. N=3 embryos, Mann-Whitney test was performed because normality or equal variance test failed. *-significant difference. Error bars are SEM.

14 transcript levels fall with age below those in the controls at both E13.5 and E15.5 (Fig 3.6).

Thus, at E13.5 quantitative RT-PCR and collagen embedded culture experiments provide evidence that *Apc* is mutated with reduction of its transcriptional levels and numbers of Apc positive cells decreased in the mutant compared to the control. However the positive immunostaining of E13.5 sections of the mutant needs further explanation because it is a reproducible result.

Thus phenotypical manifestations of *Apc* mutation appear at E12.5 when Apc immunostaining shows an obvious decrease of Apc presence in the mutant DTel. *Apc* exon 14 mRNA levels in the mutant DTel drop at E13.5 according to quantitative PCR data.

3.4 Apc and Wnt

3.4.1. Beta-catenin

Beta-catenin is one of the key proteins in Wnt signalling. The effect of canonical Wnt signalling is dependent on the activity of beta-catenin in the nucleus (Willert et al., 1999). Also beta catenin has a role in the planar cell polarity Wnt pathway as a cytoskeletal protein as an intracellular part of tight intercellular junctions. Beta-catenin levels are tightly regulated in the cell (Morin et al., 1997). Is is well established that when *Apc* is mutated, the level of cytoplasmic beta catenin is increased (Morin et al., 1997).

Immunostaining for beta catenin in the cortex of the controls revealed the presence of the protein concentrated at the cell membranes at E10.5 (Fig. 3.7 a, b).

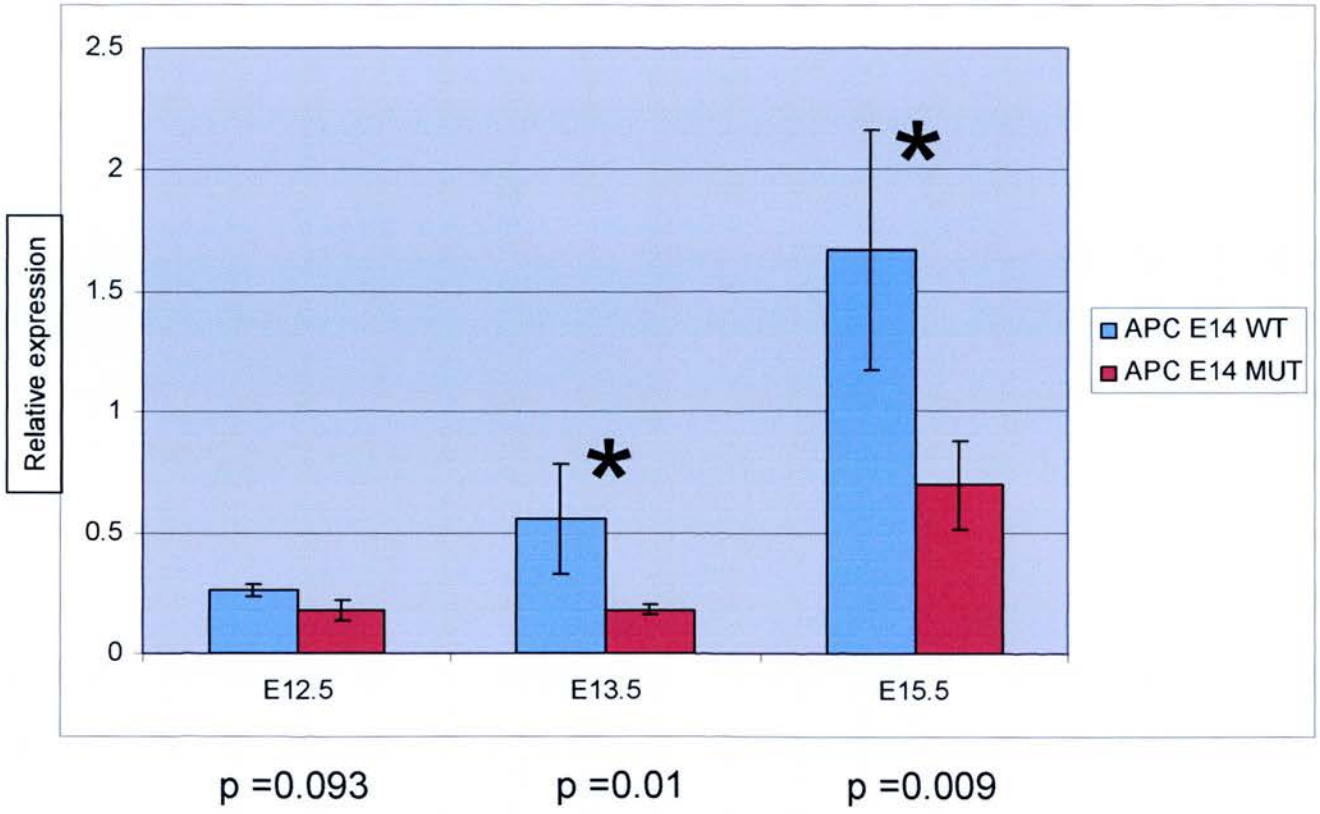


Fig 3.6 Quantitative RT-PCR of *Apc* transcript containing exon 14 in the telencephalon of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5 and E15.5. There is no significant difference in *Apc* transcript containing exon 14 expression at E12.5. At E13.5 and E15.5 there is a significant reduction of transcript containing exon 14 expression in the mutant compared to the control. N=3embryos, t-test test performed. *-significant difference. Error bars are SEM.

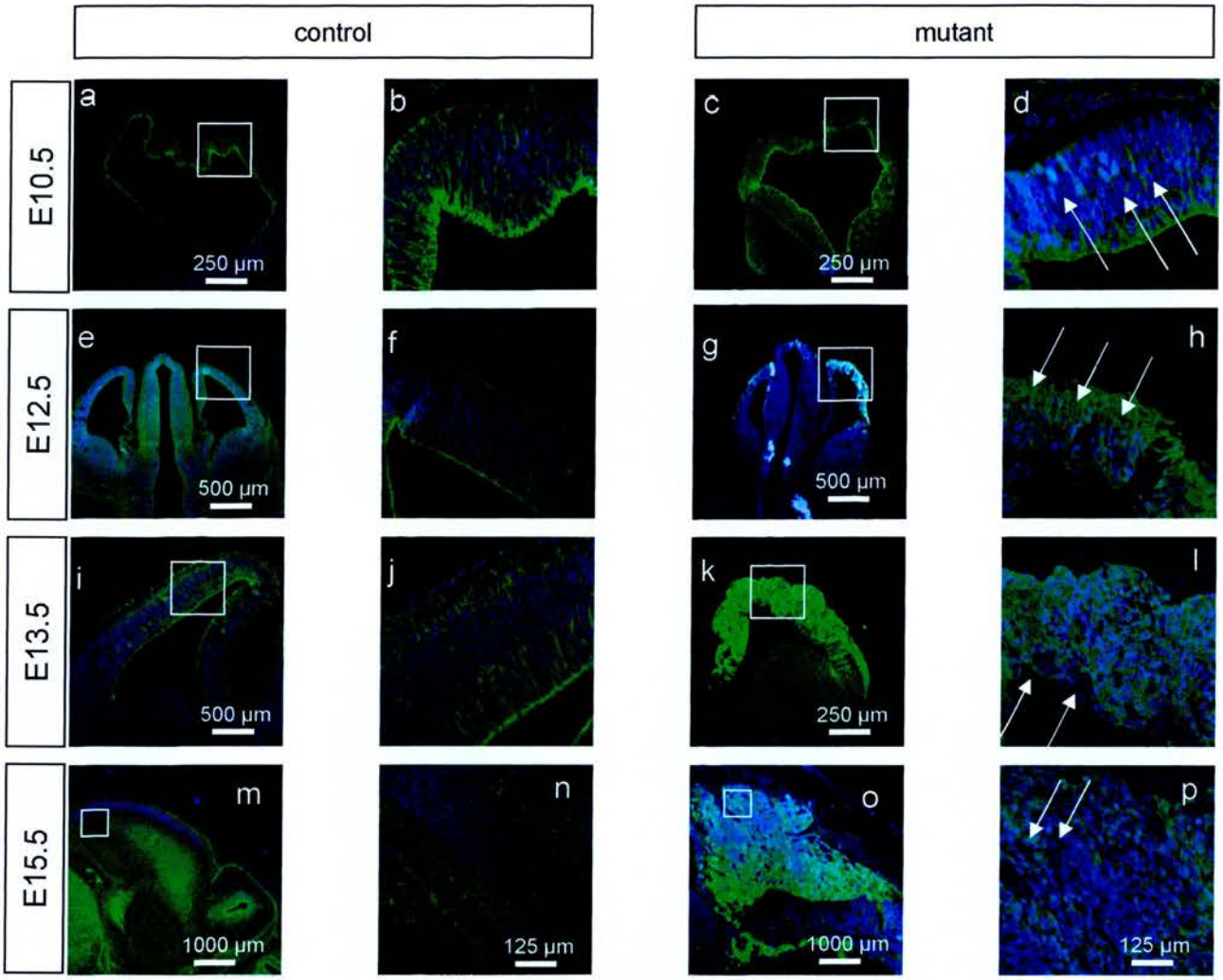


Fig 3.7 Progression of nuclear beta-catenin (green) accumulation in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E10.5, E12.5, E13.5 and E15.5. In the control beta-catenin staining is present on membrane only at all stages (a and b, e and f, i and j, m and n). The mutant DTel has single positive cells at E10.5 (c, d, white arrows). At E12.5 nuclear beta-catenin positive cells are located in upper layers of the mutant DTel (g, h, white arrows). At E13.5 nuclear beta catenin positive cells are throughout the mutant DTel (k and l), however there are nuclear beta-catenin negative cells (l, white arrows). At E15.5 the mutant DTel consists of a mixed population of nuclear beta-catenin positive (p, white arrows) and negative cells (p). Blue colour – nuclear ToPro-3 staining.

However the mutant also has single cells clearly showing nuclear localization of beta-catenin (Fig. 3.7 c, d). This is the first defect found in the mutant tissue after *Apc* deletion. Beta-catenin staining remains at cellular membranes in the control at later ages: E12.5, E13.5 and E15.5 (Fig 3.7 e and f, I and j, m and n, respectively). At E12.5 the mutant reveals more nuclear beta-catenin positive cells located predominantly in upper layers of the DTel occupying more then half of the thickness (3.7 g, h, white arrows). One day later most of the cells are positive for nuclear beta-catenin, occupying more than two thirds of the thickness (Fig 3.7 k) with some nuclear beta-catenin negative cells (Fig 3.7 l, white arrows). At E15.5 there are nuclear beta-catenin positive and negative cells, which occupy approximately similar areas in the DTel of the mutant (Fig 3.7 o, p, white arrows point to positive cells).

3.4.2 Wnts

In my $Emx1^{Cre/+}Apc^{580S/580S}$ model canonical Wnt signalling is effectively activated without proper stimulation by Wnt molecules. According to our current understanding, over-activation of the canonical Wnt signalling pathway leads to increased expression of negative Wnt regulators (Axin2, for example) (Lustig et al., 2002). However a possible change in Wnt ligand production levels after activation of the canonical pathway is not addressed in the literature to date. Therefore an interesting question is how expression of Wnt molecules changes if the Wnt signalling system is overactivated due to *Apc* mutation? Two Wnt molecules are tested: Wnt8b and Wnt1.

Wnt8b was chosen because it is expressed in the future hippocampus and the discrete regions in the dorsal thalamus (Lako et al., 1998, Richardson et al., 1999). Therefore Wnt8b is expressed in a directly affected area (hippocampus). Wnt1 is expressed in the midbrain and hindbrain regions, but not in the cortex, or more generally forebrain (Panhuysen et al., 2004). Also Wnt1 is important for kidney development (Herzlinger et al., 1994). Thus, Wnt1 provides an opportunity to find evidence of fate change.

Data on expression profiles for *Wnt8b* and *Wnt1* were obtained by quantitative RT-PCR, performed on samples of the DTel collected at E12.5, E13.5 and E15.5 from the control and the mutant embryos. *Wnt8b* shows no difference in expression at E12.5 between the control and the mutant (Fig. 3.8). With age, levels of *Wnt8b* decrease gradually in control embryos, while *Wnt8b* levels in the mutant do not drop (Fig 3.8). There is no expression of *Wnt1* in the control at E12.5, while the mutant shows the presence of *Wnt1* (Fig 3.9). At E13.5 levels of *Wnt1* are significantly higher in the mutant compared to the control (Fig. 3.9). Two days later there is no detectable *Wnt1* in the control, but there is an obvious level of *Wnt1* in the mutant.

3.4.3 Activation of Wnt target genes expression: BatGal, c-myc, cyclin-D1, p21, Axin2

Activation of canonical Wnt signalling leads to Tcf/Lef-dependent transcription of various genes (Reya et al., 2000). Therefore there is a possibility that increased levels of nuclear beta-catenin as a result of loss of Apc in the developing cortex enhance the

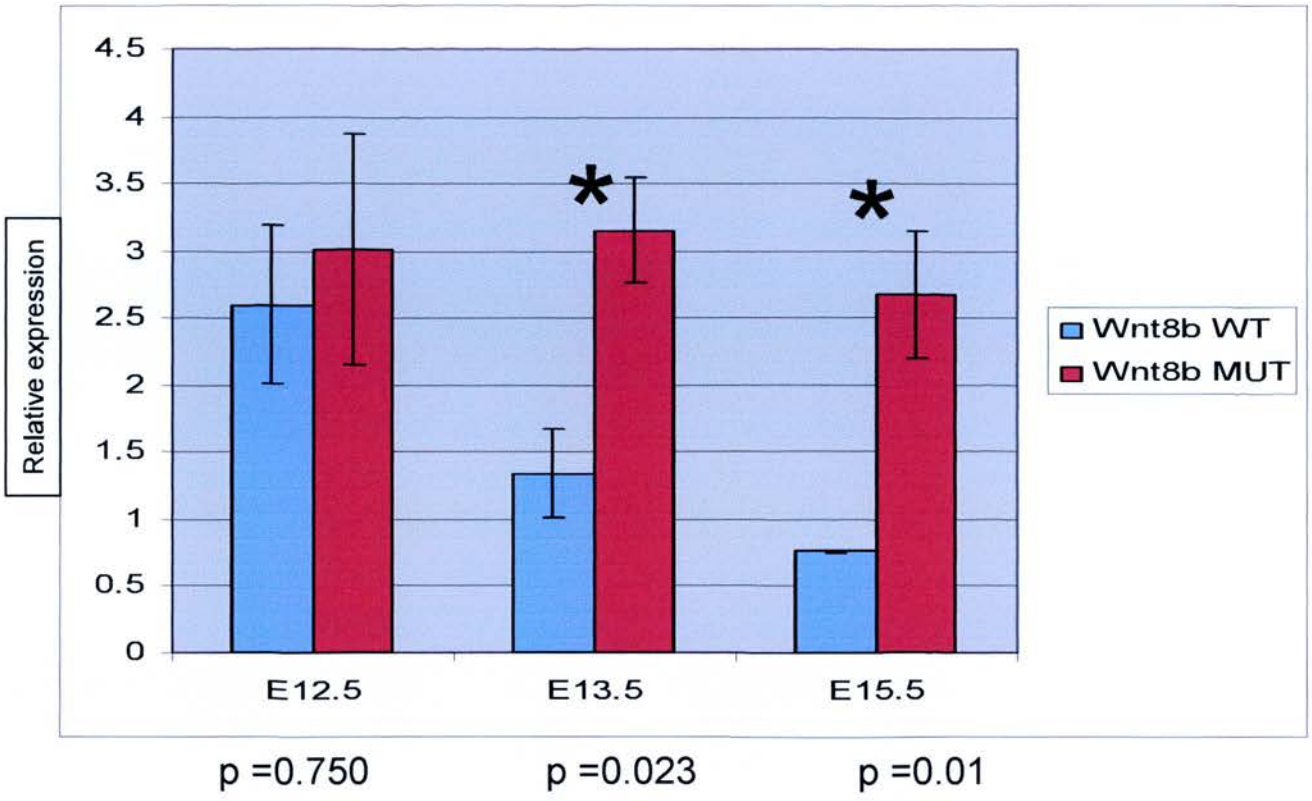
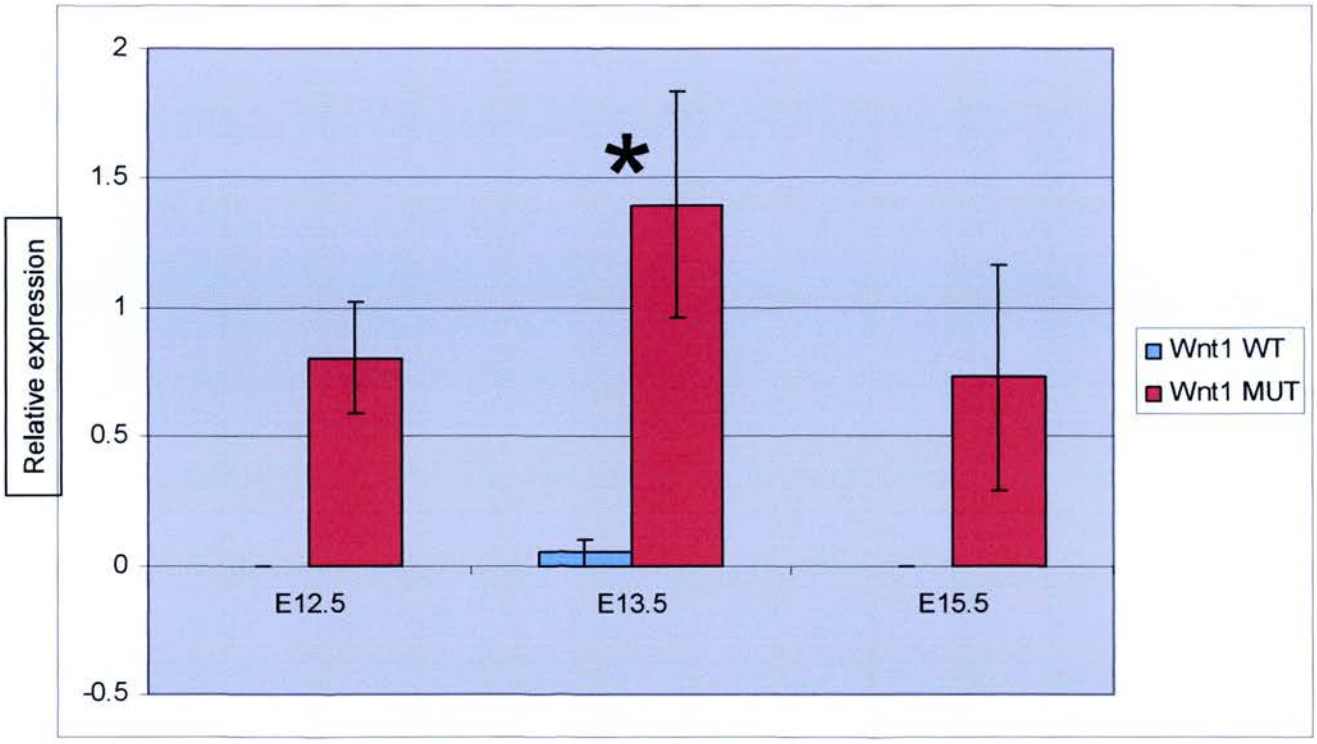


Fig 3.8 Quantitative PCR of *Wnt8b* expression in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5 and E15.5. At E12.5 levels of *Wnt8b* are similar between the control and the mutant. There is significant up-regulation of *Wnt8b* levels at E13.5 and E15.5. N=3 embryos, Mann-Whitney test performed at E12.5 because normality test failed, t-test performed at E13.5 and E15.5.*-significant difference. Error bars are SEM.



p =0.071 p =0.038 p =0.2

Fig 3.9 Quantitative PCR of *Wnt1* expression in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5 and E15.5. There is significant difference in levels of *Wnt11* expression at E13.5. However at E12.5 and E15.5 there is no statistically significant difference in expression of *Wnt1*. N=3 embryos, Mann Whitney test performed at E12.5 and E15.5 because normality or equal variance test failed, t-test performed at E13.5.*-significant difference. Error bars are SEM.

transcription of Wnt target genes. Another way to demonstrate over-activation of the canonical Wnt signalling is to use a BatGal reporter mouse line. This reporter has seven fused Tcf/Lef-binding sites and expresses beta galactosidase in response to beta-catenin specific activation (Maretto et al., 2003). A beta-catenin activity through Tcf/Lef binding is easily revealed in these mice using an enzymatic reaction with X-gal, which is converted by beta-galactosidase resulting in blue staining in locations of the enzyme activity (Maretto et al., 2003). Both the control and mutant show heavy staining in the skin of the head (Fig 3.10 a, e, i and c, g, k, respectively). Comparison of intensities of enzymatic reaction in the medial DTel reveals more intense staining in the mutant compared to the control in the rostral (Fig 3.10 d and b, respectively), middle (Fig 3.10 h and f, respectively) caudal (Fig 3.10 l and j, respectively) regions of the DTel. These results are consistent with over-activation of the canonical Wnt signalling due to beta-catenin stabilization, namely more intense staining in the mutant compared to the control.

An alternative approach to detect activation of Wnt signalling in Bat-Gal embryos is to use immunostaining for beta-galactosidase. This technique is convenient for double immunostaining when co-localization of different markers has to be tested. Unexpectedly at E13.5 immunostaining for beta-galactosidase does not show increased staining in the mutant compared to the control. Moreover, there appears to be a relative reduction of beta-galactosidase positive cells in the mutant compared to the control in caudal (Fig 3.11 d and b, respectively), middle (Fig 3.11 h and f, respectively) and rostral (Fig 3.11 l and j, respectively) regions of the DTel. Also there is an interesting staining pattern in the thalamus. The control has stripe-like staining in the dorsal

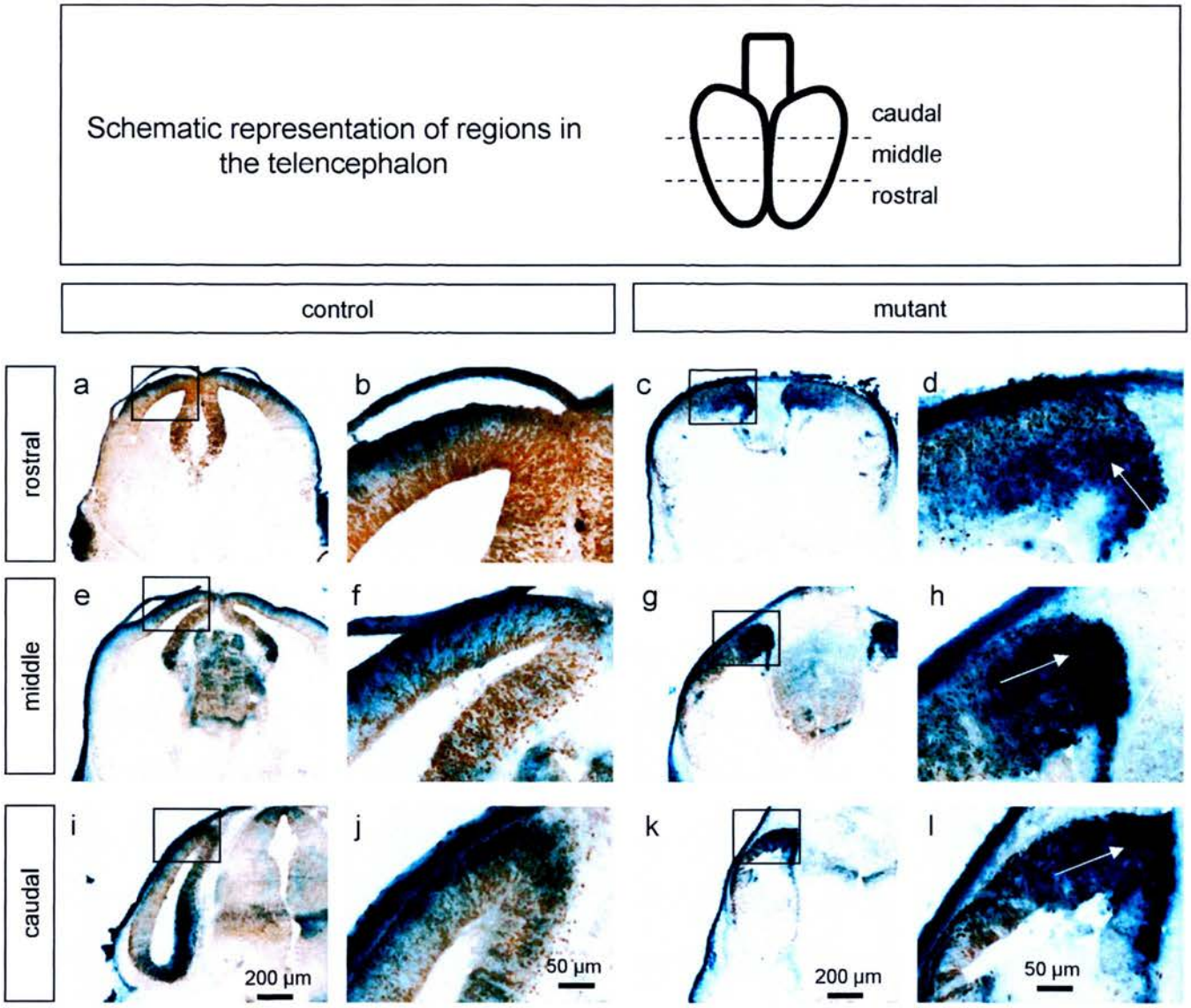


Fig 3.10 X-gal-staining (blue) for beta-galactosidase and in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 carrying Bat-Gal reporter. X-gal staining in less intense in the control than in the mutant in all areas of the DTel: rostral (a, b and c, d white arrow), middle (e, f and g, h, white arrow), and caudal (i, j and k, l, white arrow) .Brown – immunostaining for beta-galactosidase.

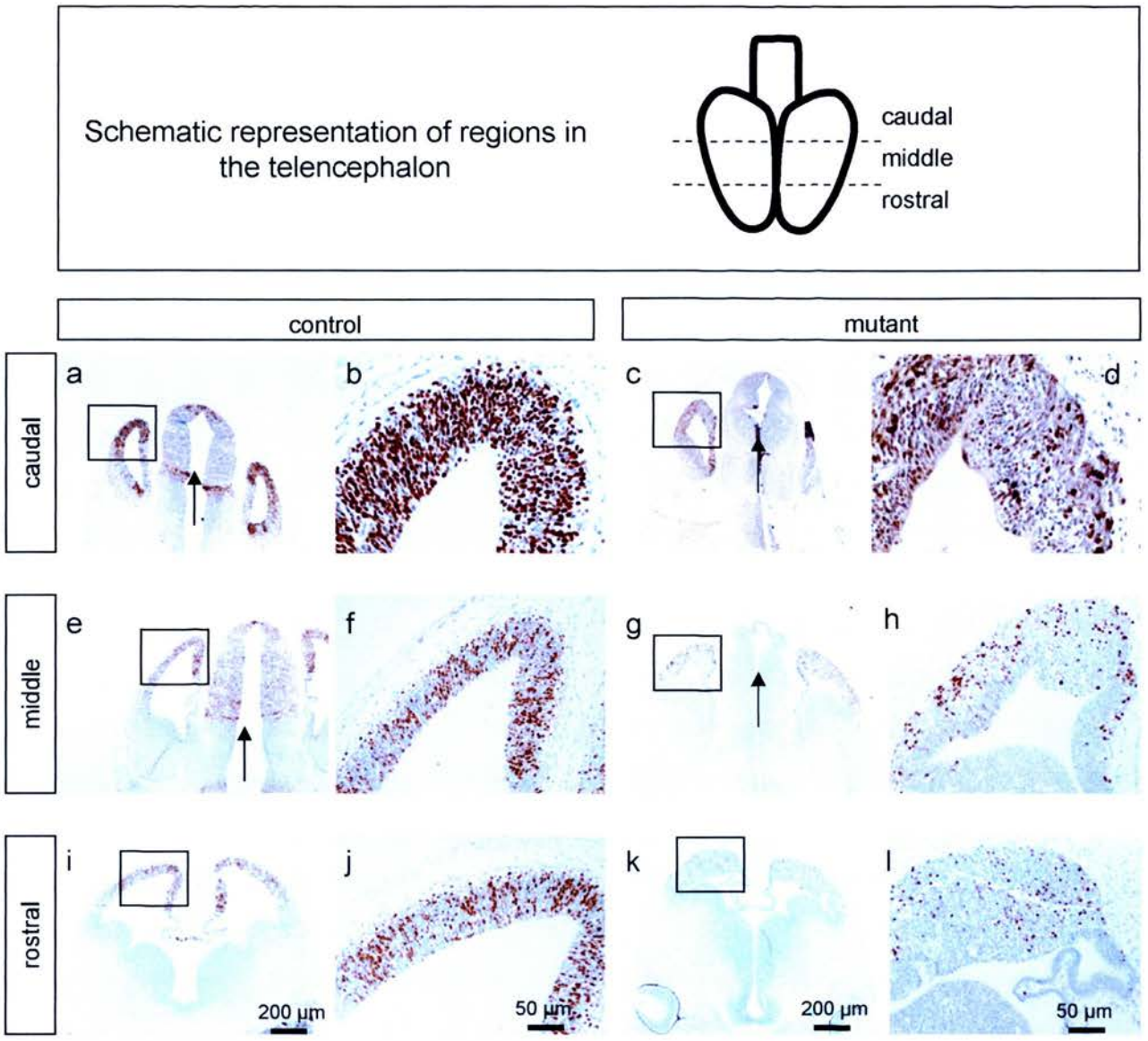


Fig 3.11 Immunostaining for beta-galactosidase (brown) in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5. Immunostaining looks less in the mutant than in control in caudal, middle, and rostral regions of the DTel. Also immunostaining is present in the thalamus of the control (a and e, black arrows) but not the mutant (c and g, black arrows). Blue colour – haematoxylin counter-staining.

thalamus (Fig 3.11 a and e, black arrows) whereas the thalamus of the control is negative for beta-galactosidase staining (Fig 3.11 c and g, black arrows).

c-Myc is one of genes whose transcription is activated by Wnt signalling (Reya et al., 2000). *c-Myc* is activated in many types of cancer (He et al., 1998). At E13.5 immunostaining for c-myc shows that there is no expression of c-myc in the control (Fig. 3.12 a and b), whereas the mutant shows expression of this protein in the DTel (Fig 3.12 c and d).

CyclinD1 expression is stimulated directly by beta-catenin (Tetsu and McCormick, 1999). CyclinD1 functions as a regulator of continued cell cycle progression (Stacey, 2003). The control shows undetectable levels of CyclinD1 expression in the DTel at E13.5 (Fig. 3.12 f), but the mutant DTel has cells which expressed CyclinD1 (Fig. 3.12 h).

P21 is a cyclin-dependent protein kinase inhibitor, which causes G1/S arrest of the cell cycle (Gartel et al., 1996). There are controversial data about changes in levels of p21 when Wnt signalling is overactivated. Fujimura et al. (2007) presented results which show that stabilized beta-catenin leads to decreased p21 expression during central nervous system development in mice. However there is evidence that p21 is up-regulated in tumours with activated Wnt signalling (Sengupta et al., 2007; Tiemann et al,. 2007). Expression of p21 in the control DTel is absent (Fig. 3.12 j) except for a small

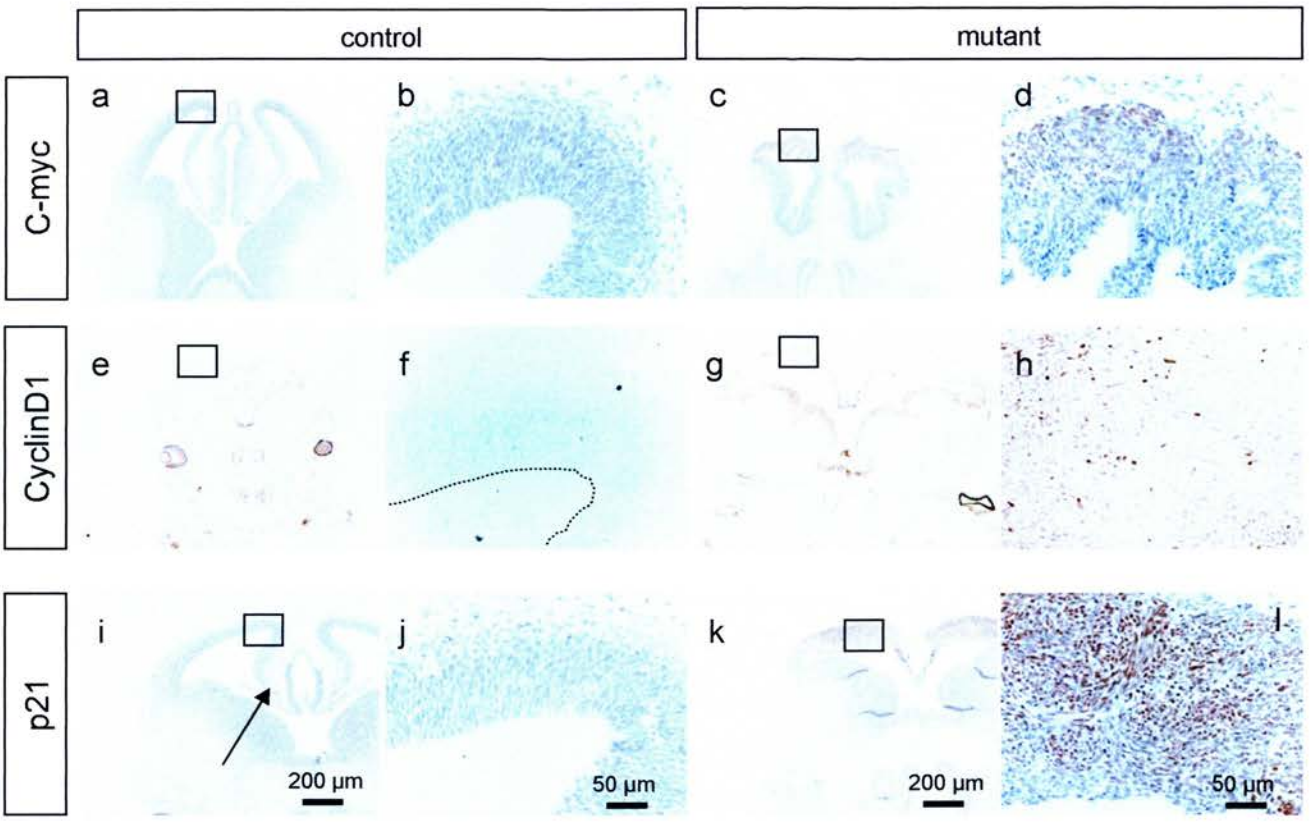


Fig 3.12 Immunostaining for C-myc (brown), cyclinD1(brown) and p21(brown) in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5. The control DTel is negative for C-myc staining (a and b), but the mutant DTel has C-myc positive cells (c and d). CyclinD1 expression is increased in the mutant DTel (h) compared to the control (f) Dotted line on f delineate the ventricular zone. P21 is up-regulated in the mutant (l) compared to the control (j). Black arrow shows p21 positive cells in the control. Blue colour – haematoxylin counter-staining.

population of cells near the hem (Fig. 3.12 i, black arrow). P21 levels are increased and detected throughout the mutant DTel (Fig. 3.12 k and l).

Axin2, or conductin, forms a beta-catenin destruction complex (Behrens et al., 1998). Axin2 expression is elevated by activation of canonical Wnt signalling (Yan et al., 2001). *Axin2* expression levels in the control DTel determined by quantitative RT-PCR remain similar through different ages (E12.5, E13.5 and E15.5) (Fig 3.13). The mutant exhibits significantly higher levels of *Axin2* at these ages compared to the control (Fig. 3.13).

3.4.4 Cell polarity and adhesion

Apc is directly involved in cell polarity as was described in Chapter 1. Also Apc has a function in cell adhesion and this function can be regulated through common with PCP Wnt pathway elements (Watanabe et al., 2004). Therefore dysfunction of Wnt signalling due to mutation of *Apc* could have an effect on cell polarity and adhesion. Also it was shown that Wnt signalling is involved in regulation of cell polarisation and cell adhesion (Strutt et al., 1997). Therefore it is a possible hypothesis that defects in polarisation and cell adhesion can be revealed during development of the mutant DTel.

Pericentrin is a centrosome protein required for proper spindle organisation (Zimmerman et al., 2004). The position of centrosomes in the cell is tightly regulated

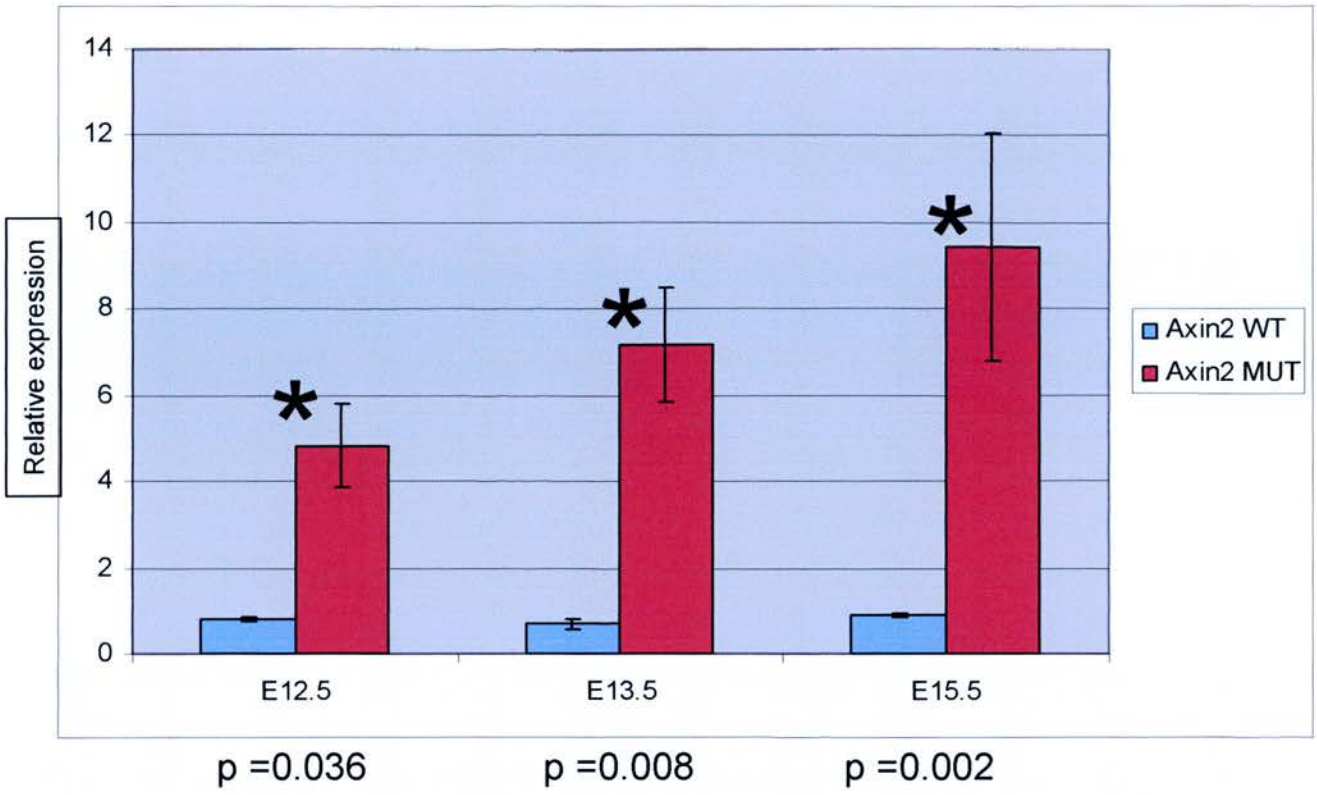


Fig 3.13 Quantitative PCR of *Axin2* expression in the telencephalon of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5 and E15.5. There is significant up-regulation of *Axin2* level at E12.5, E13.5 and E15.5. N=3 embryos, Mann Whitney test performed at E12.5 and E15.5 because normality of equal variance test failed, t-test performed at E13.5. *- significant difference. Error bars are SEM.

(Higginbotham and Gleeson, 2007). Therefore it is a characteristic marker of cell polarity. At E10.5 Pericentrin staining does not show a difference in centrosomes' distribution in the controls and the mutants (Fig. 3.14 b and d, respectively). At this age Pericentrin staining is present at the ventricular surface with a single Pericentrin positive dot in each cell throughout the thickness of the DTel in both the control and the mutant. At E12.5 most pericentrin is concentrated at the ventricular surface in the control (Fig 3.14 f). At the same age the mutant has Pericentrin distributed through the thickness of the cortex (Fig 3.14 h). One day later the control DTel preserves intense staining of the ventricular surface (Fig. 3.14 j). The distribution of pericentrin in the mutant shows complete loss of polarity in the DTel (Fig. 3.14 l).

N-cadherin is a calcium-dependent homophilic adhesion molecule (Benson and Tanaka, 1998). At E12.5 punctuate staining for N-cadherin is located around cells reflecting intercellular junctions (Fig. 3.15 b). At the same time the level of N-cadherin is decreased in the mutant (Fig. 3.15 d). Strong staining in the mutant DTel is either at the ventricular surface and in rosette-like structures - small areas surrounded by cells and with signs of basal membrane presence (shown to by white arrow in Fig 3.15 d).

To test the effect of loss of Apc on dissociated cell cultures, adhesion to different surfaces was tested for cells from E13.5 embryos. Poly-lysine, collagen, laminin, and fibronectin were used to cover surfaces for cell culturing. Fig 3.15 e-l presents results from experiments with glass covered with different substances. ToPRO-3 staining reveals nuclei of cells. The control cells have good adhesion to all of these substrates

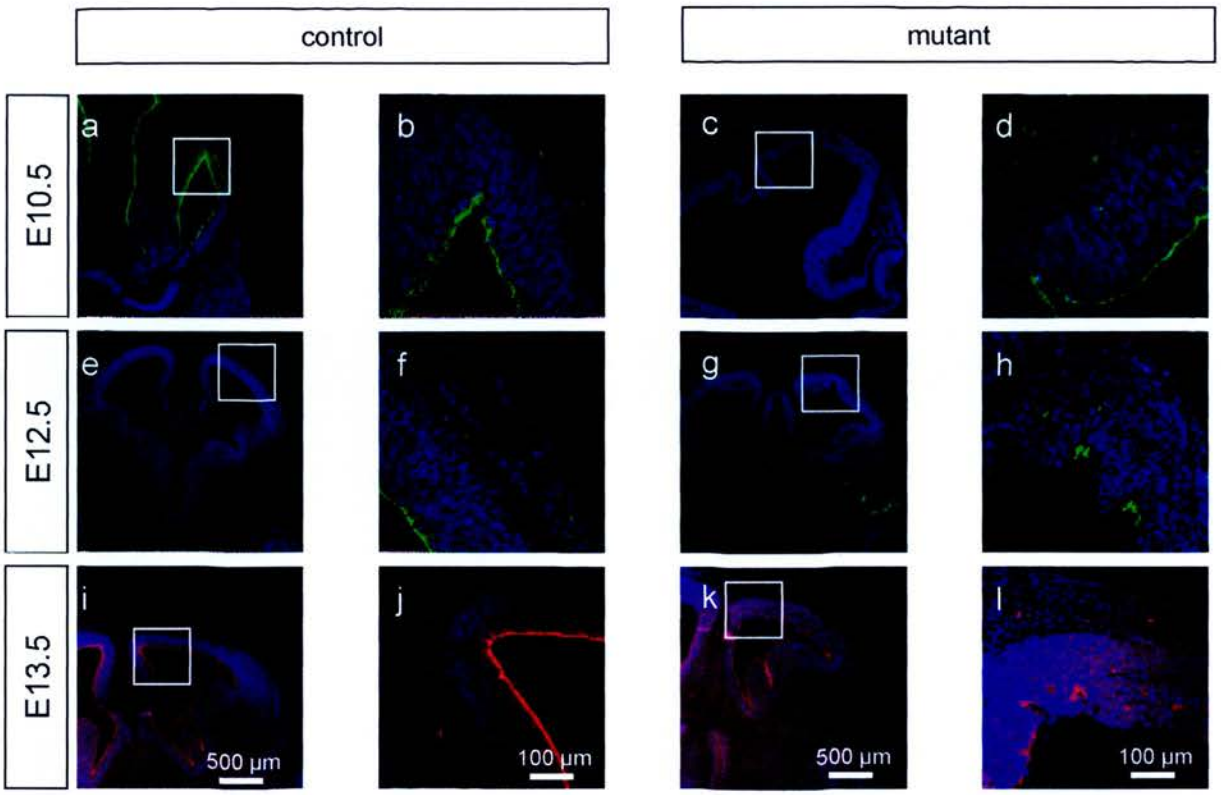


Fig 3.14 Immunostaining for Pericentrin in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E10.5, E12.5 and E13.5. At E10.5 there is no obvious difference between the control (b) and the mutant (d). At E12.5 Pericentrin is located mostly at the ventricular surface (f) in the control whereas pericentrin staining is scattered throughout the mutant DTel (h). At E13.5 the control DTel preserves continuous staining of the ventricular surface (j). The mutant has pericentrin distributed throughout the DTel (l). Pericentrin – green (a-h), red (i-l). Blue – ToPro-3 nuclear counterstaining

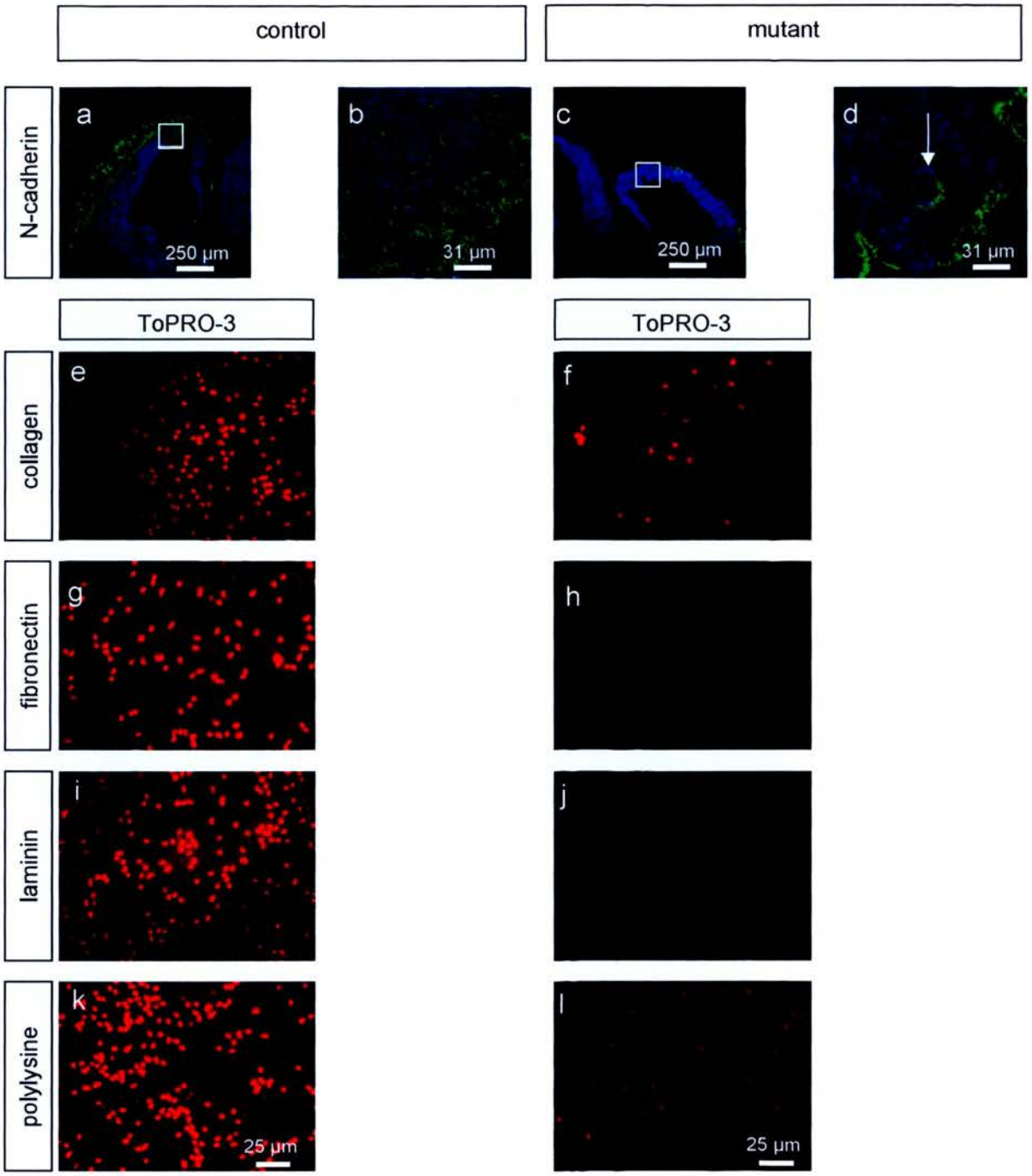


Fig 3.15 Immunostaining for N-cadherin in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5 and cell culture of E13.5 control and mutant telencephalons on different adhesion surfaces (ToPRO-3 staining). At E12.5 N-cadherin staining is decreased in the mutant (d) compared to the control (b). A rosette-like structure (a small area surrounded by cells and with signs of basal membrane presence) can be seen in the mutant (d, white arrow). There are fewer cells from the mutant DTel attached to a surface than from the control on any adhesion surface: respective sections for collagen (h and f), fibronectin (h and g), laminin (j and i), and poly-lysine (l and k). N-cadherin – green. ToPro-3 nuclear counterstaining – blue (a-d), red (e-l).

(Fig. 3.15 e, g, i, k) as can be seen by intensive red staining. However many fewer mutant cells adhered to any of the substrates (Fig 3.15 f, h, j, l). It is possible that the cells from the mutant that did adhere do not have mutant *Apc* because of incomplete *Cre* expression activation in the DTel, but this was not tested.

3.6 Other markers

Wnt signalling is involved in neuronal proliferation and differentiation (Chenn and Walsh, 2002; Hirabayashi et al., 2004) and has a broad range of other functions (Ciani and Salinas, 2005), which could be affected after Wnt signalling disregulation To further characterize the mutant DTel a few commonly used markers of differentiation and proliferation were applied to check changes in their expression and distribution in the mutant.

Nestin is an intermediate filament protein expressed in proliferating neural progenitor cells of the CNS (Gates et al., 1995). At E13.5 immunostaining of the control DTel shows that most of cells are nestin-positive proliferating progenitors (Fig. 3.16 b, e). The medial region of the mutant DTel reveals decreased nestin expression (Fig 3.16 d). The lateral region of the mutant DTel has similar nestin expression to the control lateral region (compare Fig 3.16f and e, respectively). After two days radial glial processes are revealed by nestin staining. It is possible to trace processes from the ventricular surface to the pial surfaces of the control DTel medially and laterally (Fig.

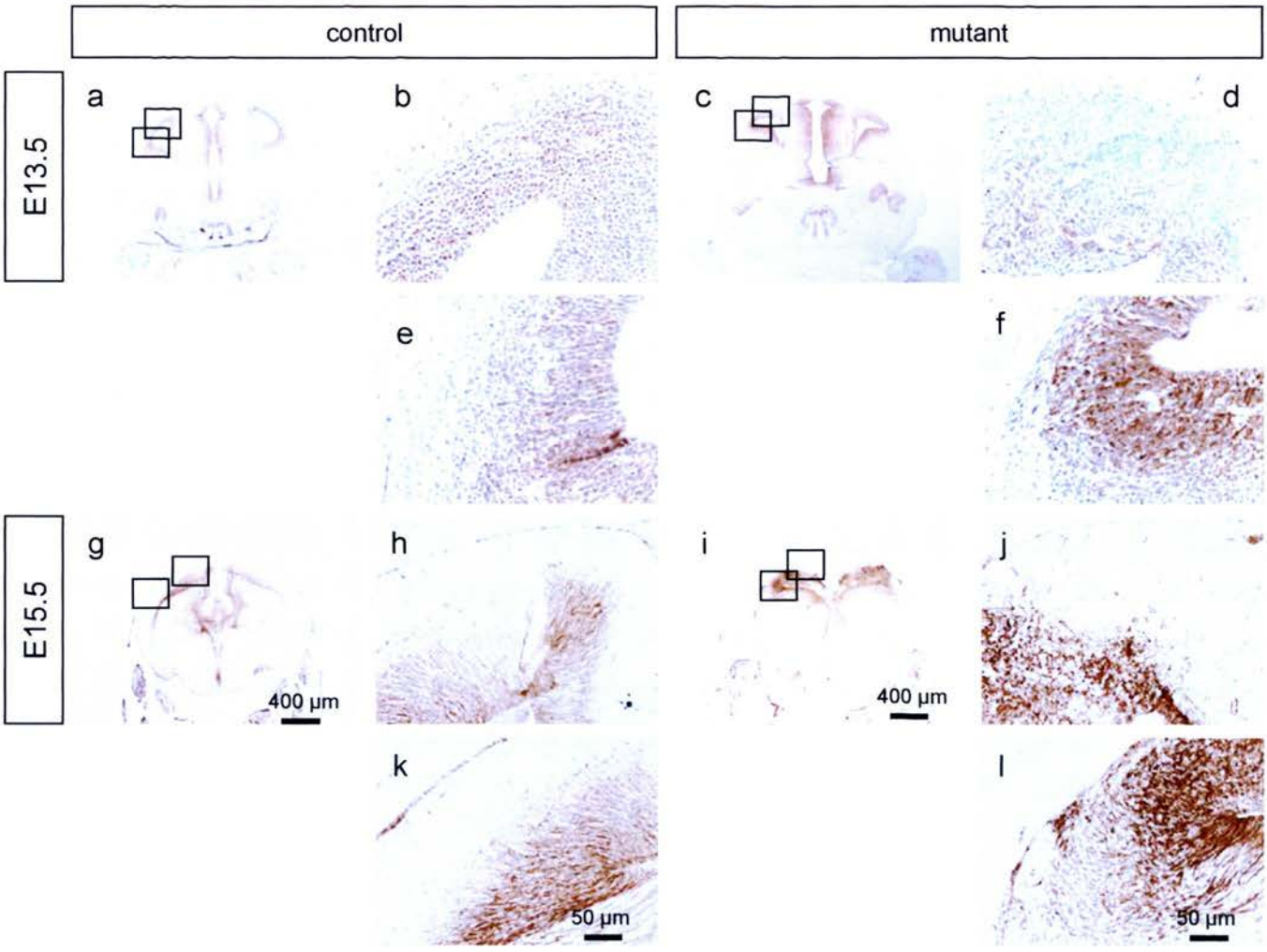


Fig 3.16 Immunostaining for Nestin (brown) in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 and E15.5. At E13.5 there is a decreased expression of Nestin medially in the control DTel (b), compared to the mutant (d). The lateral parts of the control and mutant DTels show similar expression (e and f, respectively). At E15.5 in the control DTel radial glia are arranged in palisades from the ventricular surface to the outer surface (h and k) whereas in the mutant this pattern is highly disorganised both in the medial (j) and lateral (l) parts. Blue colour – haematoxylin counter-staining.

3.16 h and k, respectively). At the same age the mutant has no apparent organisation of process growth in the DTel either medially or laterally (Fig. 3.16 j and l, respectively).

Beta-tubulin III (Tuj1) is a neural form of beta-tubulin, which is expressed by postmitotic neurons (O'Rourke et al., 1997). At E13.5 there are no postmitotic neurons in the germinal zone in the control DTel medially and laterally (Fig 3.17 h, k, respectively). Also postmitotic neurons compose roughly one third of the thickness of the medial DTel (Fig 3.17 h). The mutant DTel has TuJ1 positive cells in upper layers predominantly, both medially and laterally, but positive cells can be found in all zones of the DTel (Fig 3.17 j and l). TuJ1 positive cells compose more than a half of the medial DTel thickness. At E15.5 TuJ1-positive cells occupy all layers except ventricular (Fig 3.17 n, dashed lines). The mutant has weaker staining with positive cells distributed all over the DTel thickness (Fig 3.17 p).

Calretinin is a calcium-binding protein which is expressed in Cajal-Retzius cells (del Rio et al., 1995) and some tangentially migrating cells (Jimenez et al., 2002). Cajal-Retzius cells are early born neurons, i.e. may be able to differentiate before loss of Apc. At E15.5 calretinin staining marks two populations in the mutant DTel: Cajal-Retzius cells (Fig 3.17 b, black arrow) and cells in the intermediate zone, which possibly are tangentially migrating cells (3.17 e, black arrow). Calretinin positive cell of the mutant are distributed throughout the DTel (3.17 d and f), but further experiments are needed to trace the origin of these cells.

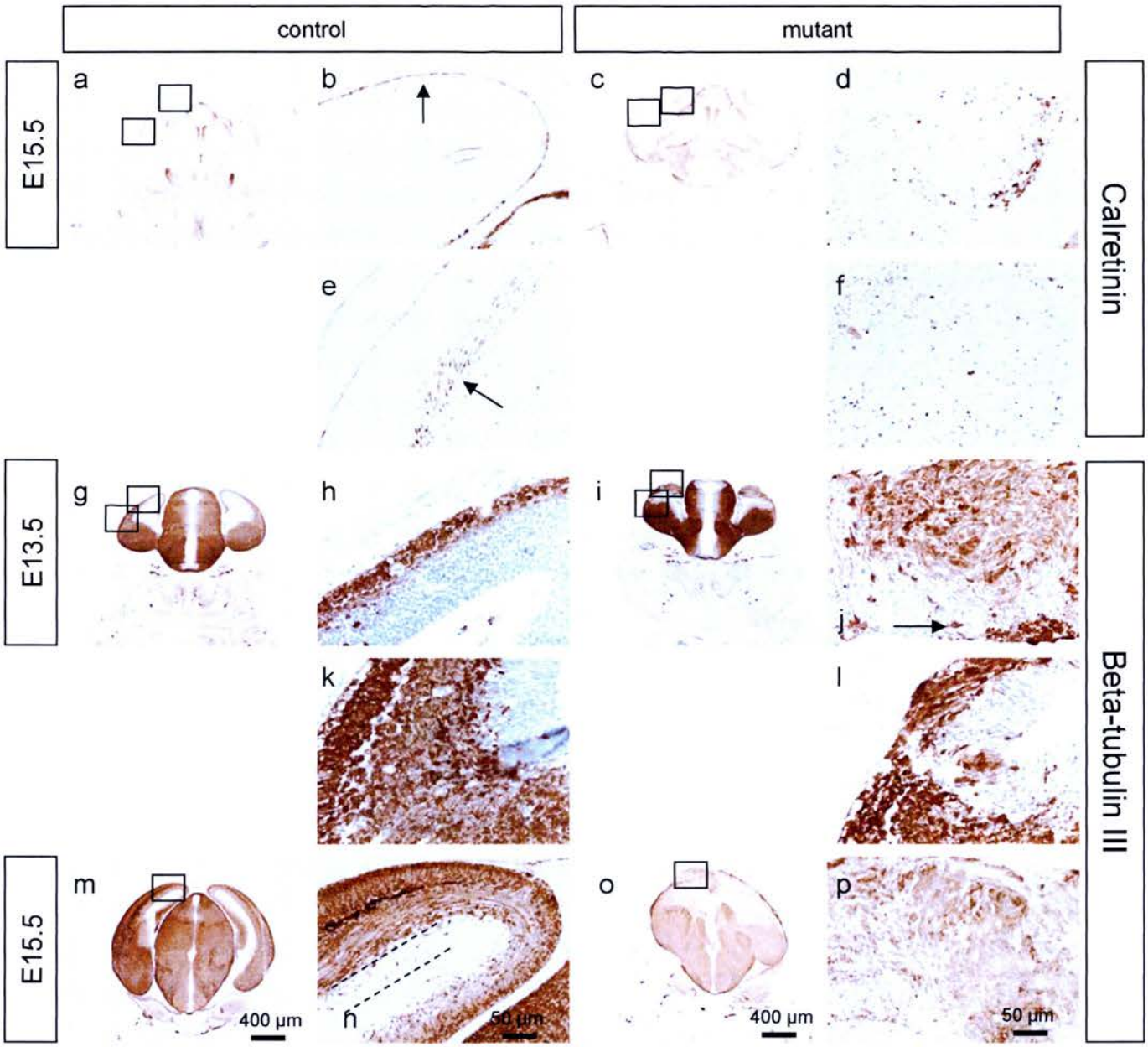


Fig 3.17 Immunostaining for Calretinin (brown) (E15.5) and beta-tubulin III (brown) (Tuj1) (E13.5 and E15.5) in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos. Calretinin stains pial surface cells of the control DTel (d, black arrow) and tangentially migrating cells (e, black arrow). In the mutant DTel calretinin positive cells are scattered throughout the DTel thickness. At E13.5 Tuj1 stains cells in the preplate (h) of the control DTel. The mutant has wider Tuj1 staining in the medial part of the DTel (j) with positive cells located in the ventricular surface (j, black arrow). At E15.5 the control has Tuj1 negative ventricular zone (n, between dashed lines). Tuj1 expression is decreased in the mutant and Tuj1 positive cells are scattered throughout DTel (p). Blue colour – haematoxylin counter-staining.

Phosphohistone3 (PH3) is a mitosis (M-phase) specific protein (Shibata et al., 1990). Staining for this protein reveals cells which are undergoing mitosis. At E12.5 most PH3 positive cells are located at the ventricular surface (Fig 3.18 b and e). A majority of PH3 positive cells of the mutant DTel do not touch the ventricular surface (Fig 3.18 d and f). One day later there are more PH3 positive cells away from the ventricular surface in the mutant, but the majority of PH3 positive cells are in the ventricular zone (Fig 3.18 h and k). Most PH3 positive cells are located in lower regions of the mutant DTel, with only single cells touching the ventricular surface (Fig. 3.18 j and l). At 15.5 the pattern of PH3-positive cell distribution in the control DTel remains similar to that of earlier stages of development (Fig 3.18 n). At the same age the mutant has scattered PH3 positive cells in the DTel, which are distributed apparently randomly throughout the thickness (Fig 3.18 p and r).

Thus, there are various defects found in the mutant at different stages. A summary of these findings in is shown in Table 3.1.

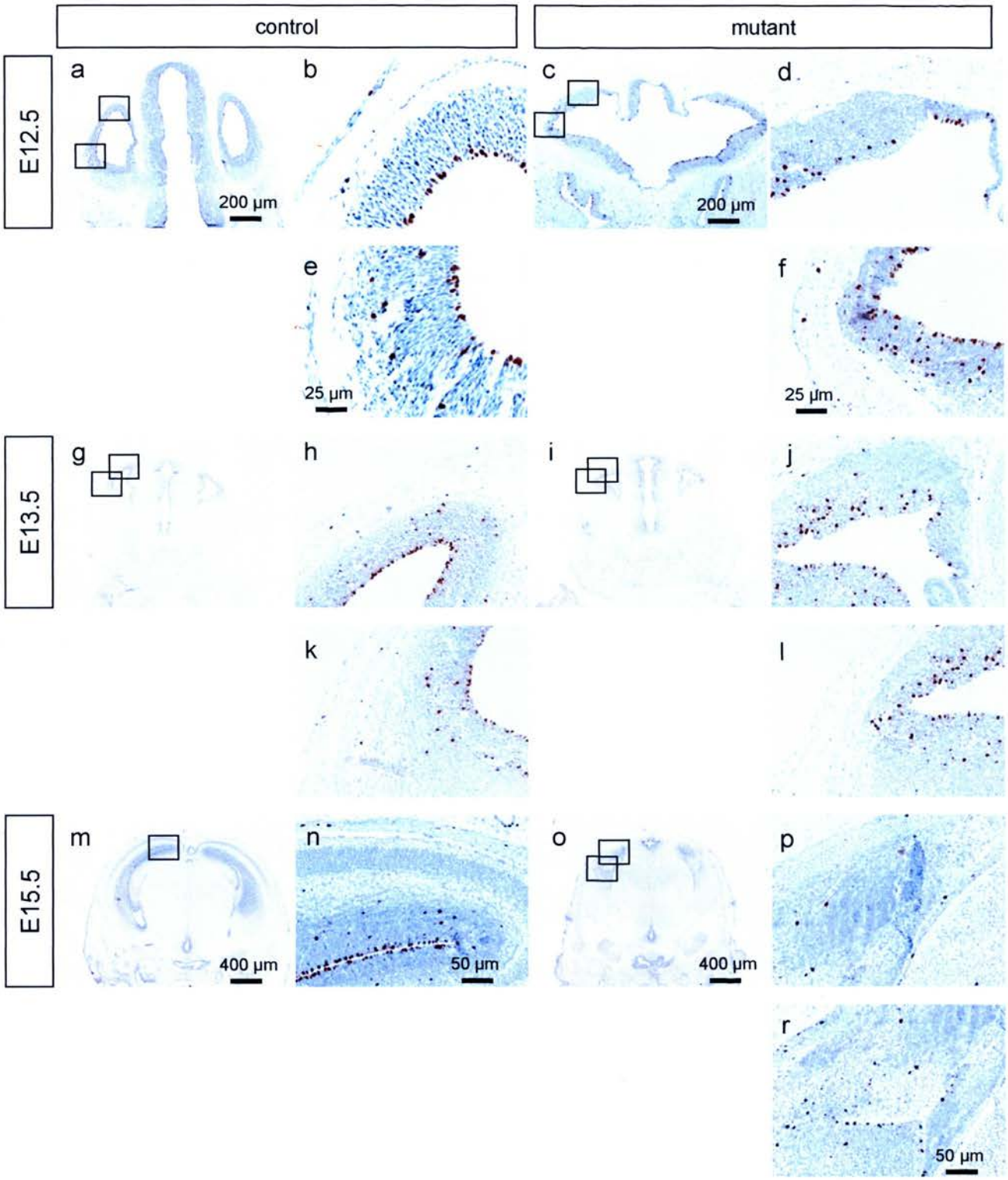


Fig 3.18 Immunostaining for Phosphohistone3 (brown) (PH3) in the telencephalon of control (*Emx1Cre/+Apc580S/+*) and mutant (*Emx1Cre/+Apc580S/580S*) embryos at E12.5, E13.5 and E15.5. At E12.5 most of PH3 positive cells of the Control DTel are located at the ventricular surface (b and e). Most PH3 positive cells do not touch the ventricular surface of the mutant (f and f). At E13.5 the control has most of the PH3 positive cells at the ventricular surface (h and k), but most PH3 positive cells in the mutant are away from the ventricular surface (j and l). At E15. 5 the control has PH3 positive cells on the ventricular surface and singular cells in the VZ and SVZ (n), while expression of PH3 in the mutant is decreased and positive cells are scattered in the DTel (p and r). Blue colour – haematoxylin counter-staining.

	E10.5	E12.5	E13.5	E15.5
Signs of *Apc* mutation	No direct signs	Negative cells for Apc	Negative cells for Apc in culture, decreased expression of *Apc*	Negative cells, decreased expression of *Apc*
Nuclear beta-catenin (not detected in the control)	Few cells	Many cells	Most cells	Many cells
Wnt8b	Not tested	Normal level	increased	increased
Wnt1(not expressed in the control DTel)	Not tested	expressed	expressed	expressed
Wnt targets (c-myc, CyclinD1, p21, Axin2)	Not tested	Elevated Axin 2, others not tested	All elevated	Elevated Axin 2, others not tested
Polarity (pericentrin)	As in control	lost	lost	lost
Adhesion	Not tested	Decreased N-cadherin staining	Decreased surface adherence	Not tested
Progenitor pool (Nestin)	Not tested	Not tested	decreased	Decreased,
Neurons (Tuj1)	Not tested	Not tested	increased	decreased
Mitosis (PH3)	Not tested	Ectopic expression	Ectopic expression	Ectopic expression, decreased expression

Table 3.1 Summary of phenotype characterization of *Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$ dorsal telencephalon at E10.5, E12.5, E13.5 and E15.5.

3.7 Discussion

3.7.1 Apc deletion

I have shown that *Apc* is mutated in the DTel of the $Emx1^{Cre/+}Apc^{580S/580S}$ embryos in regions where Emx1 is normally expressed (Fig 3.3 and Fig 3.1 c). *Emx1* has a gradient of expression in the DTel: higher medially and caudally, lower laterally and rostrally (Bishop et al., 2002). It is likely that Cre expression driven by the *Emx1* promoter has a similar pattern of expression as the *Emx1* gene. Therefore it is possible that effectiveness of *Apc* deletion will show a similar gradient to *Emx1* expression: higher caudo-medially, lower rostro-laterally. Histological examination showed that defects are more severe medially than laterally: at E12.5 medial regions look more affected than lateral (Fig 3.2 g, h). A similar high medial low lateral gradient is maintained in later stages (Fig 3.2 k, l, o, p). Apc immunostaining shows that more Apc is lost medially than laterally (Fig 3.3 g, h, o, p). This is possibly due to a gradient of Cre-activity revealed by the Rosa26 reporter (Fig 3.1 c). However the high-caudal low-rostral gradient seems to be not enough to produce an obvious histological defect difference in the DTel, possibly due to a sharper medial-lateral gradient that caudal-rostral.

Apc immunostaining of the mutant DTel reveals that at E12.5 and E15.5 mutant tissue has Apc negative regions whereas at E13.5 the mutant DTel is all positive, however dissociate E13.5 cells show a clear decrease in Apc. This immunostaining result at E13.5 is reproducible but RNA levels of *Apc* at this age are decreased. This discrepancy needs an explanation. The half-life of Apc protein is not established.

Experiments with siRNA for Apc showed that abnormalities start when Apc level are diminished to 6-22% of their normal concentration (Dikovskaya et al., 2007). Experiments performed on the colon using Apc^{580S} showed that nuclear translocation of beta-catenin occurred on the third day after *Apc* deletion (Sansom et al., 2004). My data suggest that the first signs of beta-catenin nuclear translocation appear at E10.5, which is a day after *Emx1* induction and presumptive *Apc* deletion. Quantitative RT-PCR shows that expression of transcript containing *Apc* exon 4 is increased in the mutant at E12.5 (3 days after deletion) which might reflect compensatory up-regulation of *Apc* transcription in response to decreased level of Apc protein. At later stages there is no increase in the level of *Apc* mRNA in the mutant. Quantitative RT-PCR results for transcript containing *Apc* exon 14 reveal that the level of full length *Apc* transcript mRNA remains similar to control until E12.5 (3 days after recombination). At later stages levels of full-length *Apc* transcript mRNA drop. These data suggest that full length Apc can be produced in the mutant DTel at levels similar to control until E12.5. It is possible that close-to-control levels of full-length *Apc* at E12.5 in the mutant reflects increased expression of full length *Apc* mRNA production in those cell which still have un-recombined *Apc*. However it is not possible to rule out from quantitative RT-PCR data if the cells mutant for *Apc* produce any parts of *Apc* mRNA at all.

The first defect observed - nuclear beta-catenin translocation - is seen at E10.5, which means that one day is enough for Apc to stop functioning in some cells. Immunostaining for Apc at E12.5 shows Apc negative areas in the mutant DTel. One possible explanation could be the following. The onset of *Emx1* expression does not occur in all DTel cells simultaneously (Muzio and Mallamaci, 2003). Therefore there are

cells with or without recombination of *Apc*. Cells which have *Apc* recombined activate canonical Wnt target genes. Strong up-regulation of Axin2 supports this suggestion. With age there are more and more cells undergoing *Apc* recombination and there are cells that do not recombine *Apc*. It is possible to propose a model that cells with non-functional Apc produce Wnt signals, which cause activation of the canonical Wnt pathway in cells with functional Apc. As a negative feedback to this activation, cells with functional Apc start to express molecules which counteract the activation of the Wnt signalling (Axin 2, for example). Thus cells with functional Apc have canonical Wnt pathway overactivated, leading to nuclear beta-catenin accumulation and up-regulation of expression of Wnt signalling negative feedback genes. According to this hypothesis the cells with functional Apc and nuclear localized beta-catenin should be present, which can be tested by double immunostaining for Apc and beta-catenin.

At E13.5 immunostaining for the C-terminus of Apc shows positive staining in all dorsal telencephalic tissue in the mutant. However quantitative RT-PCR results on the mutant tissue at this age show reduction of full length *Apc* mRNA. Immunostaining for the C-terminus of Apc of dissociated cells from E13.5 DTel reveals different numbers of Apc-positive cells in the control and mutant cell culture – about 40% of cells are Apc negative in the mutant samples and about 9% of cells are not stained for Apc in the control ones. Positive immunostaining for Apc at E13.5 could be due to technical problems with staining antibody and/or staining protocol, but this result is reproducible with positive controls, so I suppose that this is more likely a true staining pattern. One possible explanation for this unexpected finding could be the following. The distribution of Apc into two pools was shown by Penman et al. (2005). These two pools are

cytoplasmic and membrane with Apc exchange between them. It is possible to assume that the renewal rate of Apc in the cytoplasm is faster than that of Apc involved in cytoskeleton organization. If this is true then the first sign (except actual *Apc* deletion) of the mutation would be promotion of nuclear beta-catenin accumulation because the key function of Apc in the cytoplasm is to promote beta-catenin destruction. However it is hard to test if other defects are present at the early stage (E10.5) because they may be hard to detect assuming a large number of surrounding cells with normal properties. An additional assumption is that membrane-bound Apc does not stain with antibodies because its C-terminus is bound to other membrane proteins. Thus, if *Apc* recombination produces deficit of Apc in the cytoplasm before Apc reduction in the membrane it can be detected with Apc antibodies. There is a possibility that Apc is released from the membrane and becomes available in the cytoplasm and therefore becomes available for the C-terminus antibodies. A study of Sena and co-workers (2006) provided evidence that normal cells have strong Apc staining at membranes but light staining in the cytoplasm whereas Apc localisation in the compromised cells become moderate at the membrane and in the cytoplasm suggesting redistribution of Apc in the cell. Then cytoplasmic Apc degrades and the mutant tissue once again shows negative staining for the C-terminus of Apc.

3.7.2 Expression of Wnt targets genes

c-Myc is a proto-oncogene and one of the canonical Wnt signalling target genes (Eberhart et al., 2000). Its expression is up-regulated when the canonical Wnt pathway is

activated (Calvisi et al., 2001). A recent publication presented data on c-myc as a critical molecule of cell transformation (Sansom et al., 2007). This research group presented data that defects in phenotype in mice with *Apc* deletion are rescued by an additional *c-myc* mutation. Despite minor histological alteration in the double mutant, beta-catenin has a high level in the nucleus (Sansom et al., 2007). It was hypothesised that effects of Apc deletion are largely dependent on c-myc activity (Sansom et al., 2007). Data in this chapter show strong up-regulation of c-myc expression in the mutant DTel implying that Wnt target genes expression is activated and perhaps inducing early stages of tumour development. It was shown in pancreatic beta cell culture that c-myc is involved in apoptosis and cell cycle regulation (Pelengaris et al., 2002). Its overexpression causes an increase in cell proliferation and cell death (Pelengaris et al., 2002). Therefore it is possible to expect similar effects in the mutant DTel as c-myc is up-regulated.

P21 is a cyclin dependent kinase inhibiting protein, which controls the G1-S transition (Ball et al, 1997). It was shown in intestinal epithelial cells that an increased level of c-myc represses expression of p21, thus facilitating proliferation (van de Wetering et al., 2002). However immunostaining shows that p21 expression is up-regulated in the mutant despite up-regulation of c-myc. This might suggest that p21 is up-regulated because of a severe impairment in cellular machinery, which cause cell cycle arrest or/and apoptosis. Therefore there is a possibility that the elevation of p21 level is related to the decreased mitosis demonstrated by phosphohistone 3 immunostaining and other cell cycle markers discussed in Chapter 5.

CyclinD1 is a cyclin involved in the G1-S phase transition (McKay et al., 2000). Its concentration is increased by beta-catenin overexpression (Shtutman et al., 1999).

This is consistent with the observed immunostaining results for nuclear beta catenin and Cyclin D1 in the mutant DTel. According to the literature, activation of Cyclin D1 may lead to uncontrolled proliferation and therefore contribute to a tumour-like phenotype (Shtutman et al., 1999). Apc was first described as a tumour-related protein in colon cancers (Morin et al., 1996). Therefore Cyclin D1 up-regulation might reflect progression to a tumour phenotype.

3.7.3 Wnt signalling regulation

One major Apc function is controlling beta-catenin levels by stimulating its destruction (Clevers, 2000). Therefore an obvious hypothesis is that beta-catenin stabilisation is a predominant effect in Apc deletion. Chenn and Walsh (2002) presented data from a mouse model where beta catenin was stabilized by deleting 90 amino acids at its N-terminal part in central nervous system progenitor cells. Beta-catenin used in this experiment lacked GSK-3beta phosphorylation sites and therefore could not be destroyed in the absence of a Wnt signal. However the function of beta-catenin at adherens junctions was not affected. They found that transgenic animals with stabilized beta-catenin had an increase in the size of the brain with an enlarged surface area of the cerebral cortex. The cerebral cortex of the mutant was folded resembling the cerebral cortex of higher animals with sulci and gyri. Increased brain size was explained by expansion of the progenitor pool due to cells re-entering the cell cycle instead of differentiating. Also results from marker analysis showed that different populations of

cells are distributed normally (Cajal-Retzius cells occupy the outer layer of the DTel, postmitotic neurons (Tuj1 stained) are outside the VZ).

Beta-catenin staining of the $Emx1^{Cre/+}Apc^{580S/580S}$ mutant DTel shows its nuclear localisation. Nuclear localization of beta-catenin is related to activation of canonical Wnt target genes (Sansom et al., 2004). Axin2 is a negative regulator of the canonical Wnt signalling as it promotes beta-catenin degradation (Behrens et al., 1998). It has been shown that tumours with high beta-catenin activity have an elevated level of Axin2 (Koch et al., 2004). Results of Axin2 quantitative RT-PCR from DTel at E12.5 show a significant increase in the mutant (Fig 3.13). This is indirect evidence of Wnt signalling activation due to beta-catenin stabilization. The results obtained with the Bat-Gal reporter (Fig 3.11) support a suggestion that the activity of beta-catenin is increased in the mutant DTel. It might be expected that the effects of beta-catenin stabilization in 580S *Apc* mutation would be similar to those of the stabilized beta-catenin phenotype described by Chenn and Walsh (2002). However an overview of the 580S *Apc* mutant reveals significant differences. The DTel is misshapen and its size is not increased. The mutant tissue in the DTel is disorganized and has no obvious histological structure. At E15.5 it is possible to see spindle-like cells in the mutated area. Additional marker analysis shows that patterns of marker distribution are disrupted (this is most obvious at later stages - E15.5): Cajal-Retzius and postmitotic neurons occupy inappropriate areas within the mutant DTel. The Nestin staining pattern suggests that the proliferative pool is decreased, which is the opposite to the findings of Chenn and Walsh (2002) in their model with stabilized beta-catenin. Also additional evidence of decrease of the proliferative pool is provided by expression of the mitosis marker phosphohistone 3. Its

expression in the mutant DTel decreases with age. Thus canonical Wnt signalling activation in the mutant does not lead to a phenotype similar to that in mice with stabilized beta-catenin as described by Chenn and Walsh (2002). In their work beta-catenin was able to perform its functions in the adherens junctions and the *Nestin* promoter defined the area of beta-catenin stabilization. Therefore this suggests that stabilisation of beta-catenin is not the only factor in development of the mutant phenotype. There are other consequences in 580S Apc mutation.

3.7.4 Cytoskeletal changes

It is known that Apc has functions in cytoskeleton organization (Nathke et al., 1996). A series of experiments to clarify the interactions of cytoskeletal proteins during cytoskeleton reorganization were carried out by Watanabe et al. (2004). A Vero or COS7 cell culture model was used. They found that migration was decreased in both cell types with depleted levels of Apc. Further experiments showed that Apc depletion prevents formation of cortical actin filaments. Also it was found that Apc was important for stabilization of microtubule plus ends (the tips of microtubules where they elongate) and that Apc activity was required for microtubule organization centre (MTOC) reorientation. Experiments in which truncated forms of Apc were expressed or Apc levels were depleted using siRNA showed that migration and polarisation of cells are disrupted, providing evidence that Apc is essential for these processes (Watanabe et al., 2004). Migration and polarization mediated by Apc depend on the activity of small Rho GTPases - Rac1 and Cdc42. Rac1 and Cdc42 are downstream of the PCP Wnt pathway

(Chen et al., 2006). Besides migration and polarisation it was shown that the PCP pathway is involved in dendritic arborisation (Rosso et al., 2005). There are reports showing that mice having mutations of *Celsr3* (Tissir et al., 2005) or *Frizzled3* (Wang et al., 2002), which lead to disruption of PCP pathway, exhibit similar to each other phenotype: severe defects in development of the corticosubcortical, thalamocortical, and corticospinal tracts and of the anterior commissure. However there are no obvious defects in cell proliferation in the ventricular zone and cell migration into the developing cortex in these mutant. *Apc* mutation produces a phenotype, which is very different from that of *Celsr3* or *Frizzled-3* mutations. This suggests that a disruption of PCP pathway has a minor role in development of the $Emx1^{Cre}Apc^{580S/580S}$ phenotype.

Further investigation of the 580S *Apc* phenotype uncovers some details about cytoskeletal organization. Nestin is an intermediate filament protein and is located in the cytoskeleton (Yang et al., 2001). Immunohistochemistry shows that the mutant experiences problems with elongation of cell neurites. Processes are short and curved. This suggests that microtubules are not stabilized by Apc and therefore long neurites can not form. Votin et al. (2005) presented data which showed that Apc was required to form and elongate neurites. Also this group showed that stabilized beta-catenin inhibited membrane protrusions and extensions.

The observed polarity defect in the Apc mutant might be due to defects in reorientation of the MTOC, as inhibition of this process occurs when Apc is depleted after siRNA transfection or truncated forms of Apc are expressed (Watanabe et al., 2004). Also establishing the polarisation of neurons requires Apc dependent transport of mPar3, an axonal specifying molecule, to the tip of the axon (Shi et al., 2004). C-

terminus mutation of Apc leads to defects in neuronal polarity (Shi et al., 2004). Also there is a connection between Cdc42 localisation and distribution of Par6, a protein which is important for asymmetric cell division (Joberty et al., 2000). Later it was found that Cdc42 regulates cell polarity through regulation of Apc and GSK-3beta (Etienne-Manneville and Hall, 2003). The influence of Apc mutation on the symmetry of cell division was not tested. Phosphohistone 3 staining shows that the mutant DTel has more cells which undergo mitosis away from the ventricular surface. This phenomenon might reflect defects in polarity, when cells lose their orientation, or/and cytoskeletal defects, when cells are unable to migrate.

3.7.5 Effect on cell adhesion

Full length Apc is indispensable for proper cell adhesion in colon cancer cells (Faux et al., 2004). There is a connection between the level of beta catenin and cadherin. Apc deletion leads to stabilisation of cytoplasmic/nuclear beta-catenin. Stabilized beta-catenin becomes available for TCF/LEF binding and activation of expression of target genes. It was shown that nuclear beta-catenin down-regulates expression of cadherin (Jamora et al., 2003). The mutant DTel exhibits decreased levels of N-cadherin in the cytoplasmic membrane. The punctate staining pattern characteristic of the control is lost in the mutant (Fig 3.15 b and d). Beta-catenin has a role in adherens junctions by providing a link between cadherins and the cytoskeleton (Ohkubo and Ozawa, 1999). It was found that there is a decreased level of membrane-localized beta-catenin in the mutant. A possible explanation of these results could be that excessive cytoplasmic beta-

catenin translocates to the nucleus, where it activates the transcription of certain genes and down-regulates expression of cadherins. Cadherin levels drop down in the cell. Thus beta-catenin has less opportunity to bind cadherins and therefore more beta-catenin remains in the cytoplasm. Adhesion defects are very profound because experiments with dissociated cells show very weak adhesion of the mutant cells to a variety of surfaces, whereas the control cells adhere to any given surface roughly equally (Fig 3.1 e-l). It is possible to hypothesise that adhesion defects are secondary to canonical Wnt signalling activation if it is assumed that reduction of N-cadherin level is the result of its expression down-regulation by stabilized beta-catenin.

3.7.6 Wnt ligands

Wnt8b is expressed in the developing forebrain. Regions of expression include future hippocampus and areas in dorsal thalamus and hypothalamus (Lako et al., 1998, Richardson et al., 1999). Expression of *Wnt8b* in the germinative neuroepithelium throughout neurogenesis suggests that this protein may be important for cell proliferation (Lako et al., 1998). Wnt8b works through the canonical Wnt pathway (Saitoh et al., 2002). There is a hypothesis that Wnt8b participates in morphogenesis of the choroid plexus and medial pallium in chicks (Garda et al., 2002). Also it was shown that over-activation of Wnt8b is involved in posteriorizing forebrain structures in zebrafish (Kim et al., 2000). Therefore increased expression of Wnt8b in the mutant DTel might reflect both dorsalizing and posteriorizing of DTel tissue.

As was shown in over-expression experiments on zebrafish both wnt1 and wnt8b had a role in forebrain posteriorizing (Kim et al., 2000). Wnt1 utilizes the canonical Wnt pathway (Chou and Howard., 2002). Brain expression of *Wnt1* is restricted by the midbrain-hindbrain boundary and is crucial for proper development of the midbrain-hindbrain region (Panhuysen et al., 2004). Wnt1 expression is present throughout the CNS midline except in the telencephalon and cortical hem (Ligon et al., 2003). It was found also that ectopic expression of Wnt1 increases proliferation of progenitor cells (Panhuysen et al., 2004). Also there is an observation that Emx2 repression of Wnt1 expression in the DTel is essential for proper development of the cortical preplate (Ligon et al., 2003). This group showed that Wnt1 expression was enough to cause defects in formation of the marginal zone in the cortical plate. The appearance of Wnt1 expression in the mutant DTel suggests that there is a possibility that Emx2 expression is lost as specification of the mutant DTel is affected (more details in chapter 4). However expression of Wnt1, but not Emx2 repression, is necessary to produce all defects observed by Ligon et al., 2003. There is a possibility that strong activation of canonical Wnt signalling after Apc deletion has a powerful posteriorizing activity which overcomes Emx2 expression.

Expression of Wnt8b and Wnt1 in the mutant DTel leads to a hypothesis that midline structures are expanded as these two genes are important for their development (Lako et al., 1998, Ligon et al., 2003). However the Wnt1 expression pattern in the mutant DTel suggests that up-regulation is more related to the posteriorizing effect of beta-catenin stabilization.

3.7.7 Summary

In conclusion it was hypothesised that phenotype of 580S *Apc* mutation develops due to stabilization of beta catenin and would resemble that described by Chenn and Walsh (2002). However $Emx1^{Cre/+}Apc^{580S/580S}$ phenotype is drastically different from that with stabilized beta-catenin. Apc has other function besides promotion of beta-catenin degradation. Therefore 580S *Apc* mutation phenotype reveals over-activation of canonical Wnt pathway and disruption of the cytoskeletal related functions of Apc with a loss of the apical-basal polarity of the progenitor cells.

4. APC and Specification (Patterning)

4.1 Introduction

Proper development of the brain is a result of a coordinated action of many signalling pathways (Wilson and Rubenstein, 2000). Wnt signalling is one of them. One important component of the Wnt signalling pathway is beta-catenin, whose concentration is tightly regulated. Apc is a protein of the beta-catenin destruction complex (Behrens et al., 1998). In the presence of Apc beta-catenin is phosphorylated and subsequently destroyed (Behrens et al., 1998). The 580S mutation of *Apc* prevents beta-catenin degradation. This leads to beta-catenin accumulation in the cells that can simulate canonical Wnt signalling activation (Iwamoto et al., 2000). Local suppression of Wnt signalling activity is essential for specifying the telencephalon from anterior neural ectoderm (Houart et al., 2002). Therefore it is expected that changes in activity of canonical Wnt signalling may alter patterning of the affected area. There are gradients of canonical Wnt signalling activity in the brain (Nordstrom et al., 2002 and Backman et al., 2005). First is the anterior-posterior (A-P) gradient (high posterior, low anterior) and the second is dorso-ventral (D-V) gradient (high dorsal, low ventral). These gradients are established before the neocortex is specified (Nordstrom et. al., 2002, Lee et al., 2000). If these gradients are altered it is logical to expect shifts of expression of markers related to anterior-posterior and dorso-ventral patterning. Besides patterning shifts there is a possibility that the neuronal tissue with over-activated Wnt signalling may change identity to other tissue identities as canonical Wnt signalling is important for other organ

development (Herzlinger et al., 1994). Here these suggestions are tested by deleting *Apc* in the DTel from E9.5 and examining expression of a panel of A-P, D-V and non-neuronal tissue markers.

It is not possible to match regions/layers of the control dorsal telencephalon to those of the mutant dorsal telencephalon, because the mutant tissue is so severely affected (Fig 3.1). Therefore instead of the ventricular zone (VZ) the term ventricular zone* (VZ*) is used to describe the area of the mutant cortex which corresponds most closely to the wild type VZ based on location close to the ventricle. Activation of the Wnt signalling pathway could lead to a change in cell fate. To test this possibility, I compared the expression of a large panel of regionally expressed protein markers in control and mutant brains.

4.2 Telencephalic marker (Foxg1)

There is evidence that Wnt signaling affects the development of telencephalic tissue (Houart et al., 2002). Dorsal telencephalon properties might change during development if Wnt signaling is deregulated. Therefore the telencephalic marker Foxg1 was used to see if tissue normally possessing expression of this marker is present in Apc mutants. Foxg1 is a winged helix transcription factor expressed in the telencephalon and anterior neural component of the eye, with exception of the roof plate and the cortical hem (Xuan et al., 1995). Telencephalic progenitor cells can be recognized by Foxg1 expression from E8.5 (Dou et al., 2000).

$Emx1^{Cre/+}APC^{580S/580S}$ animals show alteration in Foxg1 expression with age. At E10.5 expression in the mutant ($Emx1^{Cre/+}APC^{580S/580S}$) (Fig. 4.1 d, e, f) is similar to that in the control ($Emx1^{Cre/+}APC^{580S/+}$) (Fig. 4.1 a, b, c). Both mutant and control tissue have decreased expression of Foxg1 in the telencephalic roofplate (Fig. 4.1 black arrows in e and b, respectively). Foxg1 expression is found throughout the control telencephalon after further development (E12.5) (Fig. 4.1. g, h, i) whereas expression is lost in the medial region of the mutant dorsal telencephalon (Fig. 4.1 k). The lateral part of the mutant dorsal telencephalon still expresses Foxg1 at E12.5 (Fig. 4.1 l). At E13.5 Foxg1 expression remains in the dorsal telencephalon (DTel) of the control (Fig. 4.2 a, b, c). At this age the mutant has single cells expressing Foxg1 in the DTel medially (Fig. 4.2 e, black arrows). There are Foxg1 positive and Foxg1 negative regions in the lateral region of the mutant dorsal telencephalon (Fig. 4.2 f). A corresponding control region has a much thicker Foxg1 positive layer (Fig. 4.2 c). At a later stage (E15.5) the control dorsal telencephalic tissue maintains expression of Foxg1 (Fig. 4.2 g, h) while the majority of cells in the mutant dorsal telencephalon do not express Foxg1 (Fig. 4.2 i, j).

Thus, the mutant DTel changes pattern of Foxg1 expression from E12.5.

4.3 Cortical markers: (Pax6, Tbr2, Tbr1, Ngn2)

Apc is deleted in the DTel specifically within on the *Emx1* expression area; therefore I looked at markers expressed in the DTel first. Changes of cortical marker expression patterns can provide additional information about the mutant tissue. Pax6,

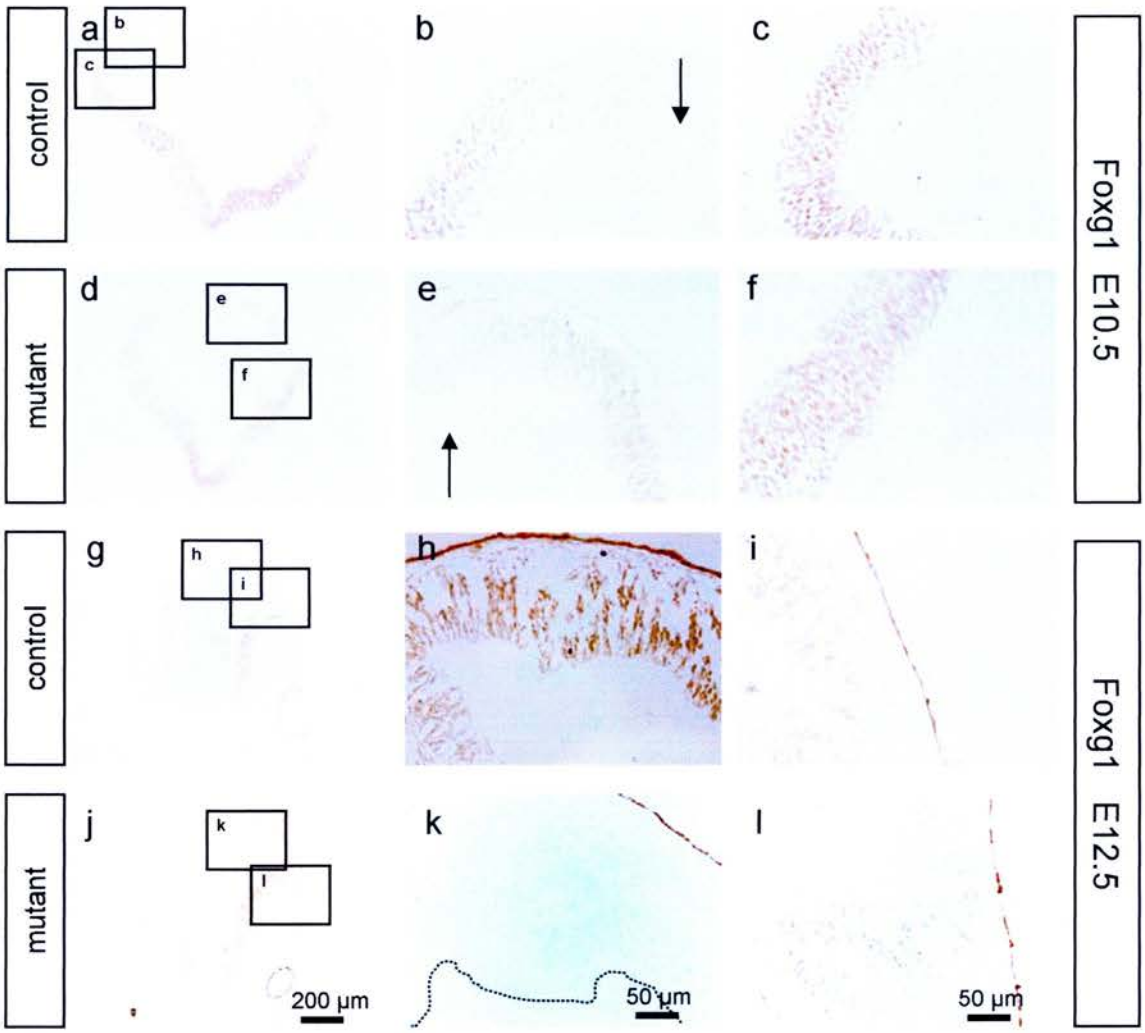


Fig. 4.1 . **Foxg1 (brown) expression in the telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E10.5 and E12.5.** E10.5 telencephalons of the control and the mutant have a similar pattern of expression (a-c and d-f, respectively). By E12.5 the control retains expression in the dorsal telencephalon (h and i) but the mutant exhibits no expression in the medial dorsal telencephalon (k) while the angle region still expresses Foxg1 (l). Dotted line on k delineates ventricular surface.

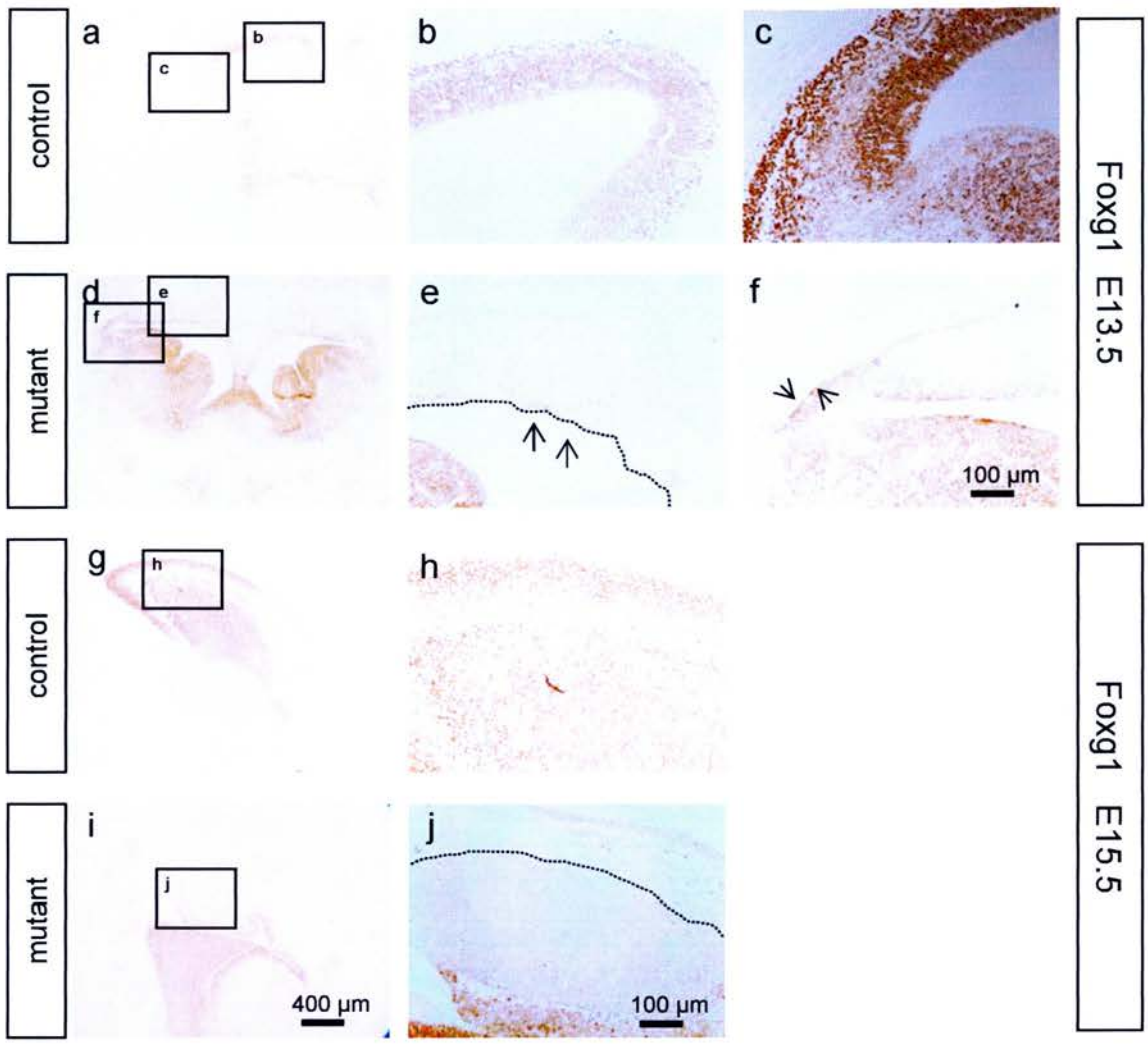


Fig. 4.2 . **Foxg1 (brown) expression in the telencephalon in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 and E15.5.** At E13.5 Foxg1 is expressed in the control telencephalon (a, b, c). The mutant telencephalon has Foxg1 expression in the ventral part (d), and angle region (f, black arrows show sizes of Foxg1 expressing and non-expressing areas). The medial dorsal part of the mutant telencephalon has a few cells expressing Foxg1 in the ventricular zone* (e, black arrows). At the later stage (E15.5) control expresses Foxg1 throughout the dorsal telencephalon (h), whereas the mutant exhibits drastic reduction in Foxg1 expression in the dorsal telencephalon (j). Dotted line on e delineates ventricular surface. Dotted line on j delineates marginal surface.

Tbr2 and Tbr1 are regionally expressed transcription factors, which are expressed by certain cell types in certain layers of the dorsal telencephalon, reflecting progression of neuronal maturation in the dorsal telencephalon. Cortical expression of Ngn2 is specific to the progenitor population.

Pax6 is a homeodomain transcription factor. Pax6 is expressed at a high level in the ventricular zone (VZ) of the cortex where mitosis takes place (Gotz et al., 1998). Also expression of Pax6 is found in the diencephalon, eye, and cerebellum (Puschel et al., 1992; Grindley et al., 1995). At E13.5 Pax6 expression in the control dorsal telencephalon is localized to the ventricular zone (black arrow on Fig. 4.3 b and c). The mutant shows another pattern of Pax6 expression: in the medial part of the DTel cells are not organized in layers and Pax6 expression is decreased (Fig. 4.3 e). Pax6 positive layers in the lateral part of the DTel have similar thickness in the control and mutant, but the mutant DTel in this area is thinner than that of the control (Fig. 4.3 c and f). At E15.5 Pax6 expression in the control DTel is restricted to the VZ (Fig. 4.3 g and h) and forms a sharp boundary in the angle region - pallial-subpallial boundary (PSB) (Fig. 4.3. i). At this age the number of Pax6 positive cells is apparently decreased in the mutant medial DTel (Fig. 4.3 k). The angle region of the mutant has less Pax6 expression and the boundary is not so sharp as in the control (Fig. 4.3 l).

Tbr2 is a T-box transcription factor that is expressed in the cortex, mesencephalon and rhombencephalon (Kimura et al., 1999). Tbr2 is highly expressed in intermediate progenitor cells in the subventricular zone and the basal ventricular zone of

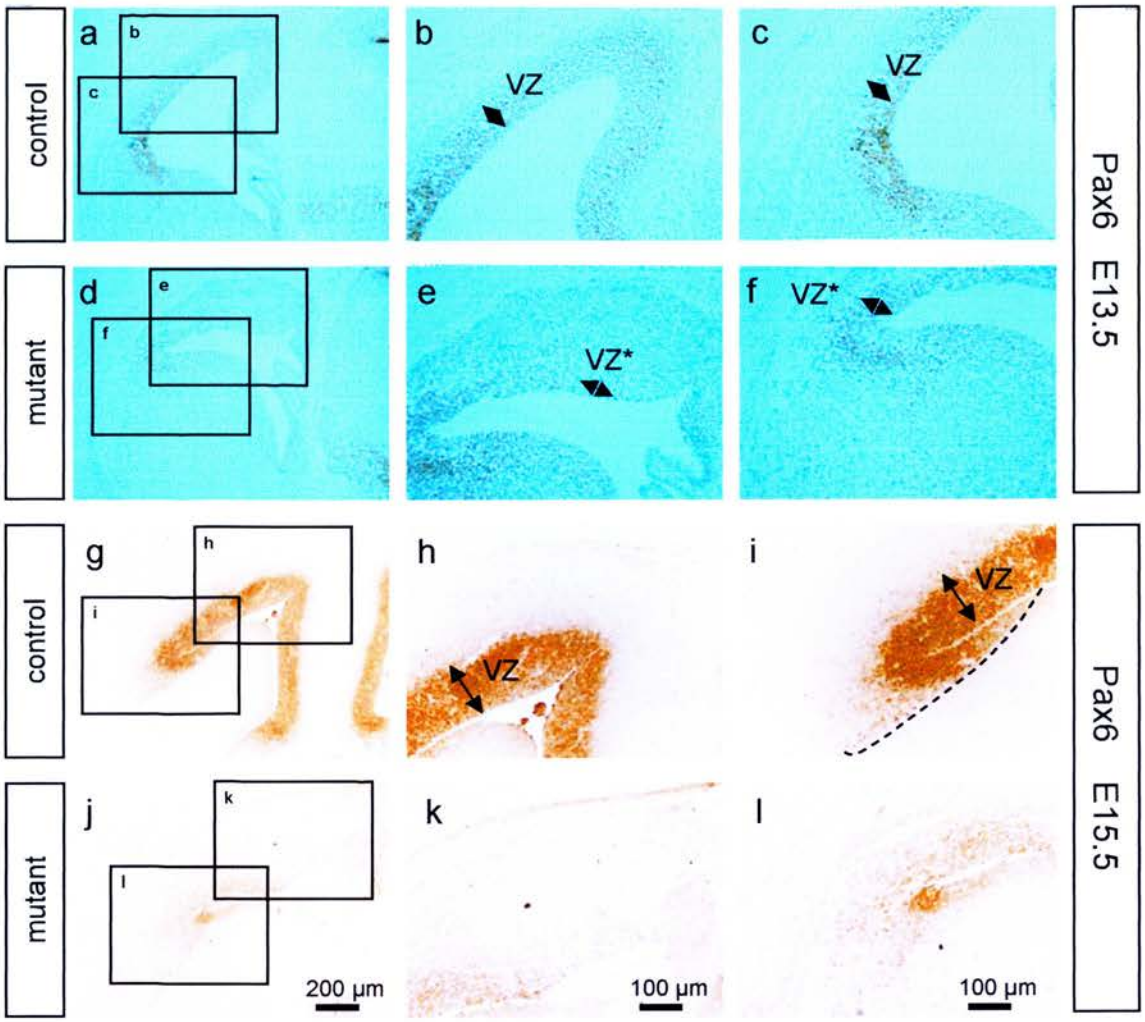


Fig. 4.3 **Pax6 (brown) expression in the dorsal telencephalon of control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos.** Pax6 immunohistochemistry of E13.5(a-f) and E15.5 brains (g-l). At 15.5 there is less Pax6 expression in the mutant (k, l) compared to the control (h, i). Expression of Pax6 is lower in the mutant both at E13.5(e) and E15.5(k) compared to respective ages of the control (b and h). The layer structure of the control (a and g) is missing in the mutant (d and j). VZ- ventricular zone. VZ*- ventricular zone in the mutant. Dashed line on I marks the pallial subpallial boundary (PSB).

the neocortex (Englund et al., 2005). At E15.5 intermediate progenitor cells form a well defined layer (predominantly in SVZ) in the cortex of the control (Fig. 4.4 a, b) with a sharp boundary in the angle region (Fig. 4.4 c). Tbr2 expression is clearly decreased in the mutant DTel. Tbr2 positive cells of the medial part of the DTel are localized in the ventricular zone* of the mutant (Fig. 4.4 e). The angle region of the mutant is also affected: cell density is reduced but the boundary is clear (Fig. 4.4 f)

Tbr1 is a T-box transcription factor that is expressed strongly in early-born cortical neurons, including subplate, layer six and Cajal-Retzius cells (Hevner et al., 2002). At E15.5 upper layers of the control DTel express Tbr1 (Fig. 4.4 g, h, i). There are alterations in the expression pattern in the mutant DTel. In the medial part the mutant expression pattern is reversed: upper layer cells are Tbr1 negative but the ventricular zone* contains Tbr1 positive cells (Fig. 4.4. k). Expression of Tbr1 is decreased. Tbr1 positive cells are scattered throughout the DTel lateral region of the mutant (Fig. 4.4 l).

The basic helix-loop-helix transcription factor Ngn2 is expressed in the progenitor cells of the ventricular/subventricular zones in the cortex. The level of expression of Ngn2 is heterogeneous in a progenitor population (Parras et al., 2002). At E13.5 Ngn2 positive cells are found in the VZ of the control DTel (Fig. 4.5 b and c). Ngn2 expression stops sharply at the PSB (Fig. 4.5 c). There is no restriction of Ngn2 positive cells to the ventricular zone* (VZ*) in the mutant DTel (Fig. 4.5 e). Moreover the VZ* of the mutant has fewer Ngn2-positive cells than most dorsal telencephalic tissue (Fig. 4.5 e). The lateral region of the mutant DTel has an inverted staining pattern

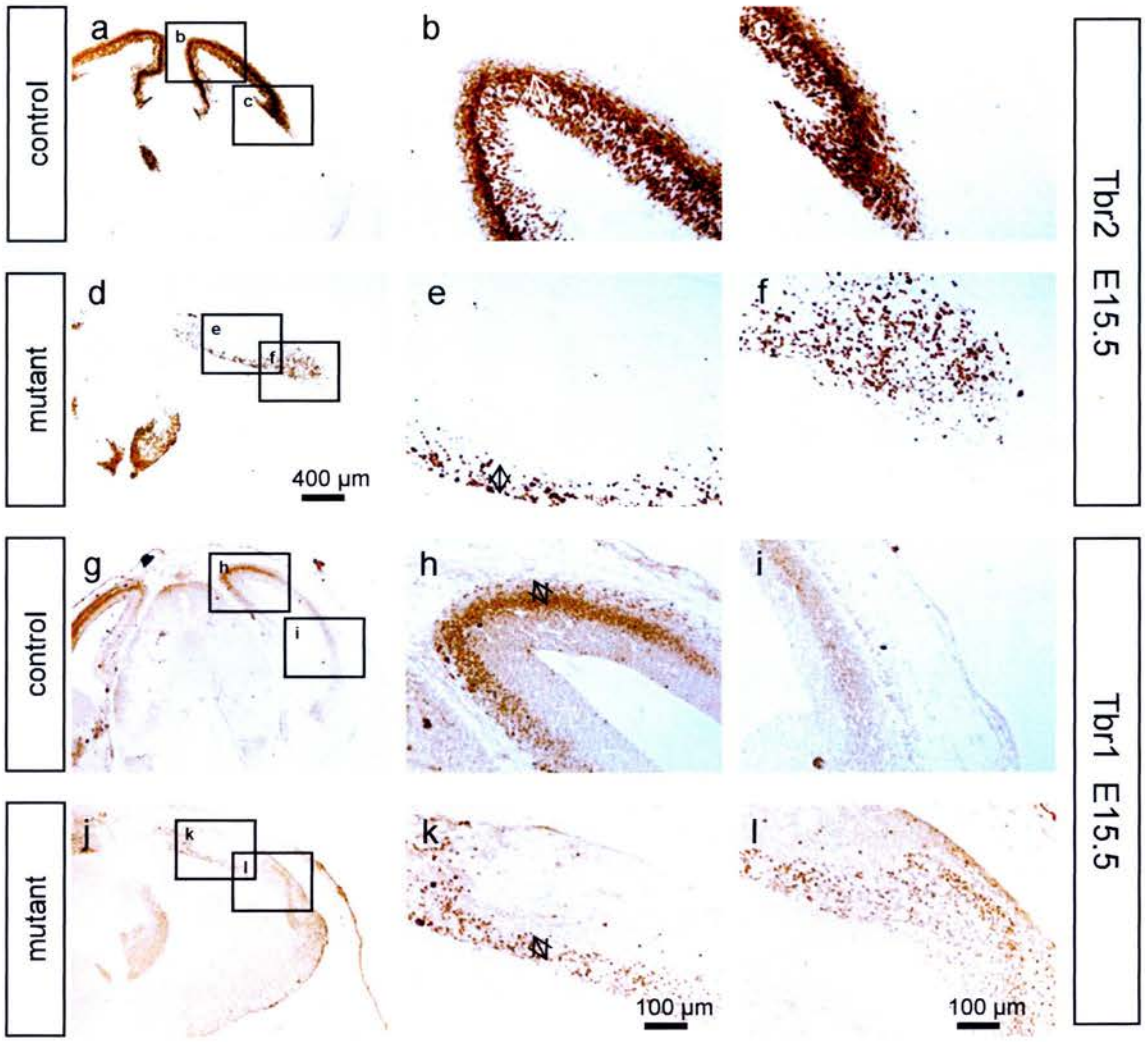


Fig. 4.4. **Expression pattern of Tbr2 (brown) (a-f) and Tbr1(brown) (g-o) in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E15.5.** Tbr2 is expressed in SVZ, basal VZ in the control (white arrow, b). In the mutant Tbr2 expressing cells are localized in the ventricular zone* (black arrow, e). There is a diffuse expression of Tbr2 in the mutant angle region (f) while the control keeps a sharp boundary (c). Tbr1 is expressed in MZ, SP, CP, IZ normally (black arrow, h). In the mutant, Tbr1 positive cells are concentrated in ventricular zone* (black arrow, k). In the angle region the control preserves a linear structure of Tbr1 expression (i) while the mutant shows diffuse distribution through the thickness (l). Both Tbr2 and Tbr1 show reduction of expression in the mutant (d and j, respectively) compared to the control (a and g, respectively).

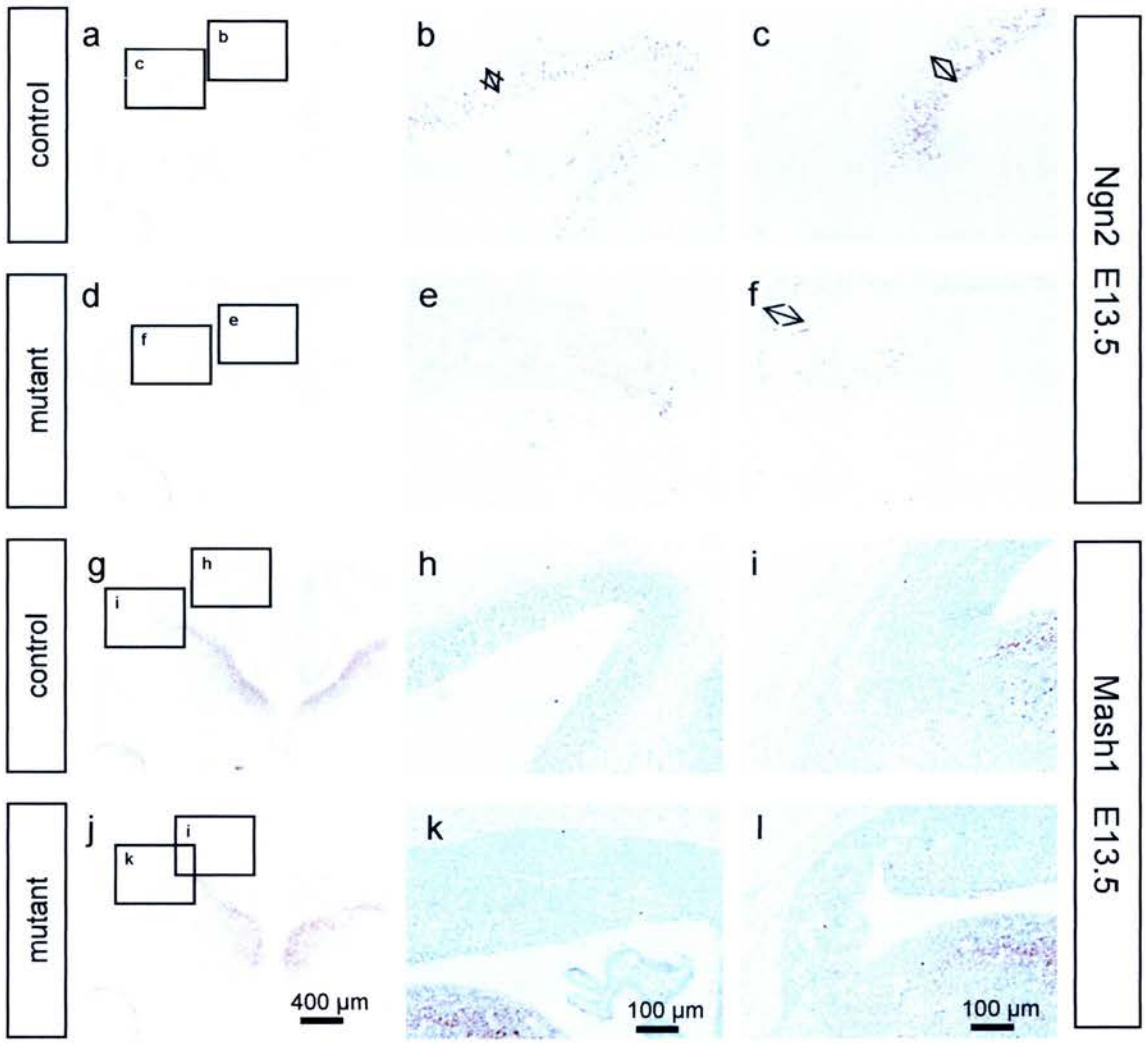


Fig. 4.5 . **Distribution of Ngn2 (brown) and Mash1 (brown) in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5.** The control cortex expresses Ngn2 in progenitor cells in the ventricular zone (b and c, black arrows). The expression pattern has a sharp boundary with the ventral telencephalon (c). Ngn2 expression in the mutant is distributed through the thickness of the dorsal telencephalon (e). The boundary is less obvious and the lateral part of the dorsal telencephalon has expression of Ngn2 in upper layers (black arrow on f) but not in VZ* (f). Mash1 distribution shows similar expression patterns in the control (g, h, i) and the mutant (j, k, l). Blue –counterstaining with haematoxylin.

for Ngn2: the VZ* is almost completely negative, but there are Ngn2 positive cells in upper layers (Fig. 4.5 e). Ngn2 expression at the PSB is also decreased (Fig. 4.5 f).

Thus, Pax6, Tbr2, Tbr1 are still expressed in the mutant dorsal telencephalon, but their distributions are inappropriate and levels of expression are decreased. Ngn2 expression is present in the mutant cortex, but its distribution is affected.

4.4 Expression of ventral telencephalic markers (Mash1, Olig2, Islet1)

Changes in expression of ventral telencephalic markers might show that ventral telencephalon is affected also. Expression of ventral telencephalic markers in the dorsal telencephalon can suggest that more cells migrating through the pallial subpallial boundary or a shift from dorsal to ventral identity.

Mash1 is a basic helix-loop-helix transcription factor. Mash1 is expressed in a subset of VZ cells and in most SVZ cells of the ventral telencephalon. Mash1 marks a population of committed neural precursors (Casarosa et al., 1999). At E13.5 there are no differences in expression of Mash1 between the control and mutant DTels (Fig. 4.5 g, i and j, l, respectively). Also there is no detectable expression of Mash1 in the medial part of DTel in the control (Fig. 4.5. h) and the mutant (Fig. 4.5 k).

Olig2 is a basic helix-loop-helix transcription factor. Olig2 is required for differentiation to motoneurons and oligodendrocytes (Mizuguchi et al., 2001). Olig2 is expressed in the ventral telencephalon (Du et al., 2006). Olig2 expression in the ventral telencephalon is present in lateral and medial ganglionic eminences of the control at E13.5 (Fig. 4.6 a). There is no expression of Olig2 in the control DTel (Fig. 4.6 b). At the same age the mutant ventral telencephalon has Olig2 expression in lateral and medial ganglionic eminences (Fig. 4.6 e). However there are single Olig2 positive cells in the mutant DTel (Fig. 4.6 f).

Islet1 is a LIM homeobox gene. Islet1 is an early marker of differentiation of the neural tube cells. Islet1 is also a marker of motoneurons in the spinal cord (Avivi and Goldstein, 1999).

There are no apparent differences in expression of Islet1 in the ventral telencephalon of the control (Fig. 4.6 c) and the mutant (Fig. 4.6 g). Both control and mutant have no Islet1 positive cells in the DTel (Fig. 4.6 d and h, respectively).

Thus, cells in the mutant dorsal telencephalon do not appear to have acquired ventral fate. Single Olig2 positive cells may reflect a compromise of the PSB.

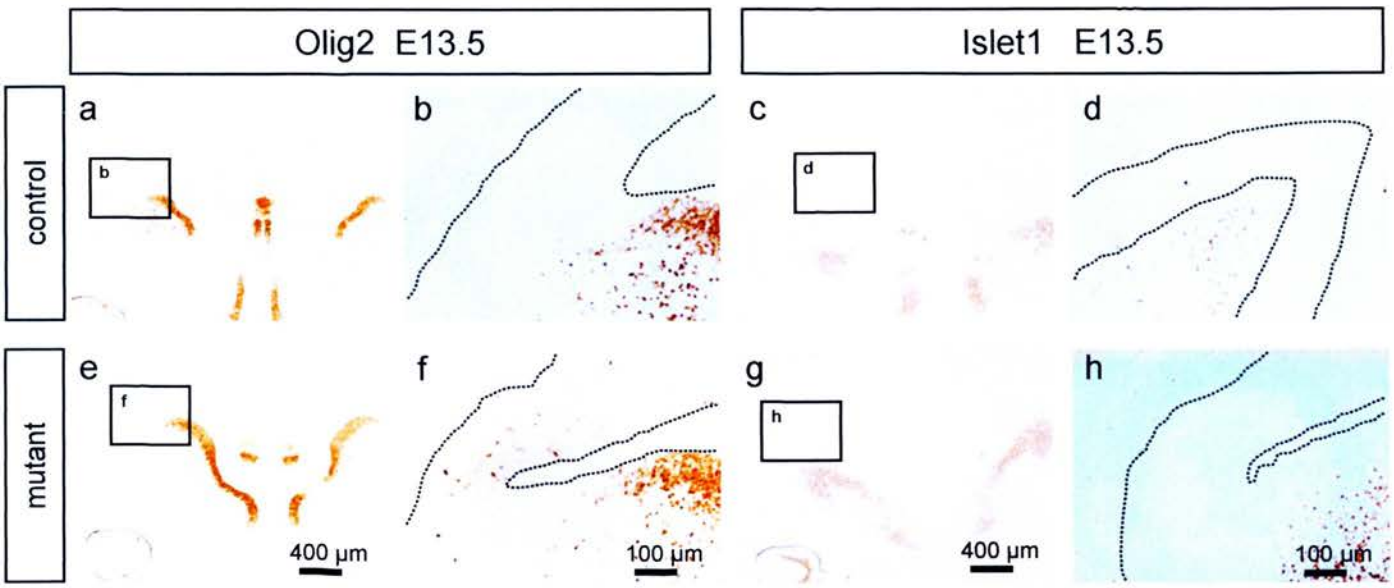


Fig. 4.6 . **Distribution of Olig2 (brown) and Islet1 (brown) expression in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5.** Most of the Olig2 expressing regions are overlapping in the control (a) and the mutant (e). There is an obvious boundary in the control (b) but the mutant has Olig2 positive cells in the dorsal telencephalon (f). There are no apparent differences in Islet1 expression in the control (c, d) and the mutant (g, h). Dotted lines on b, d, f and h delineate borders of the telencephalon.

4.5 Rostral-caudal fate (Pax3)

Pax3 is a paired box gene which is expressed in the neural tube, somites and neural crest during early embryonic development. Pax3 activity in the alar plate of the midbrain leads to differentiation of the tectum (Blake and Ziman, 2005). Pax3 is necessary for the proper formation of caudal neural crest derivatives and for the migration of myoblasts into the limb (Mansouri, 1998). Ectopic expression of Pax3 with Pax7 can induce an ectopic tectum (Matsunaga et at., 2001). At E12.5 the DTel of the control has no Pax3 expression (Fig. 4.7 b), but there is Pax3 expression in the epithalamus (Fig. 4.7 a, black arrow). Similar expression of Pax3 in the epithalamus is seen in the mutant (Fig. 4.7 e, black arrow). Also there are positive cells in the medial part of the mutant DTel (Fig. 4.7 f, black arrow) which are absent in the control DTel (Fig. 4.7 b). The control does not have Pax3 expression at the later stage (E15.5) in the DTel (Fig. 4.7 c and d). At this age Pax3 expression is greatly increased in the mutant DTel (Fig. 4.7 g). Most of the Pax3 positive cells are located in upper parts of the mutant DTel (Fig. 4.7 h).

Thus, some cells of the mutant dorsal telencephalon acquire the identity of more caudal regions than the telencephalon.

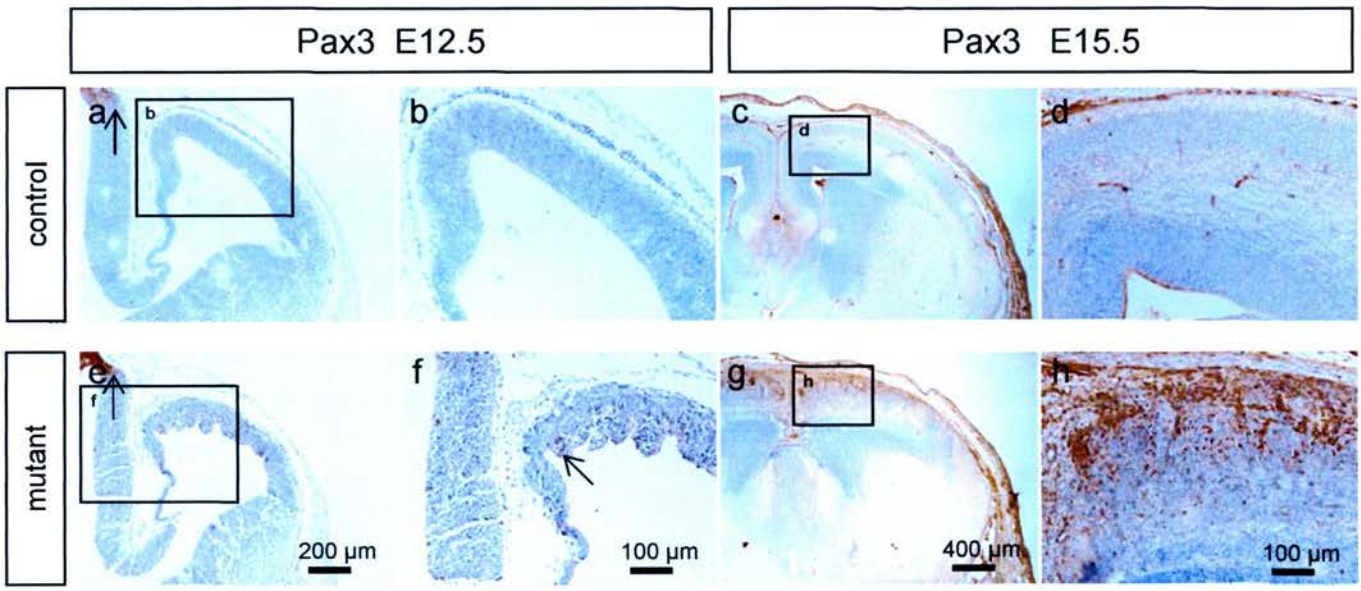


Fig. 4.7 . **Pax3 expression in the telencephalon in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5 and E15.5.** At E12.5 Pax3 positive cells are present in the epithalamus of the control (a, black arrow) and the mutant (e, black arrow). The control cortex is free of Pax3 expression (b). There are a few Pax3 positive cells in the mutant dorsal telencephalon medially (f, black arrow). At E15.5 there is no expression of Pax3 by the control cortex (c and d). The expression of Pax3 by cells is increased in the mutant dorsal telencephalon (g) and these cells are located in upper layers of the dorsal telencephalon (h). Pax3 – brown, haematoxylin - blue.

4.6 Other tissues markers (WT1, Pancytokeratins, SMA, Desmin, S100, Vimentin, MyoD1)

Given that many cells in the mutant dorsal telencephalon have unknown fate, I hypothesised that they may have adopted a non-neuronal fate; therefore a number of markers of non-neuronal fate were examined. A panel of antibodies for different tissues was applied to test alterations in the mutant tissue identity. This panel of antibodies is used in the Western General Hospital to type tumours, where immunohistochemistry was performed.

Wt1 is Wilm's tumor suppressor gene which encodes a zinc finger transcription factor. Wt1 is important for kidney, gonad, adrenal gland and spleen development (Kim et al., 2007). *Wt1* was identified as a tumor suppressor gene whose mutations are associated with the development of kidney tumors. In adult tissues, Wt1 expression is found in the urogenital system, the central nervous system and in tissues involved in haemapoiesis, including the bone marrow and lymph nodes (Yang et al., 2007). In the nervous system Wt1 is expressed in the presumptive motor neurons of the spinal cord at E11. At 15.5 there is a small domain of expression of Wt1 in the roof of the fourth ventricle of the brain (Armstrong et al., 1993).

Immunostaining for Wt1 at E12.5 did not reveal expression in the control DTel (Fig. 4.8 a and b). However there are Wt1 expressing cells in the mutant dorsal

telencephalon (Fig. 4.8 d). One day later (E13.5) the control DTel does not have obvious Wt1 expressing cells (Fig. 4.8 e and f), but there are Wt1 positive cells in the mutant DTel, which are mostly located in middle and lateral parts of the mutant DTel (Fig. 4.8 g and h). At E15.5 the control DTel has Wt1 expression in processes of neurons and the secretory epithelium of the ventricular surface (Fig. 4.8 i, j). At the same age the mutant shows cells with nuclear Wt1 localization in caudal regions of the DTel (Fig. 4.8 k and l). Also double immunostaining shows that Wt1 expression is overlapping with nuclear beta-catenin in the mutant DTel medially and laterally (Fig 4.9 e and f). The control does not have Wt1 expression or nuclear beta-catenin in the DTel medially and laterally (Fig 4.9 b and c).

Cytokeratins are intermediate filaments, expressed in the skin and choroid plexus. Cytokeratins have names from K1 to K20 (Gu and Coulombe, 2007). An antibody cocktail containing monoclonal antibodies to K5, K6, K8, and K18 was used for immunohistochemistry. These monoclonal antibodies were designed to recognize almost all epithelial tissues and their neoplasms. At E13.5 Pancytokeratin expression is found in choroid plexus of both control (Fig. 4.10 a, b) and mutant (Fig. 4.10 e). There is no expansion of Pancytokeratin expression into the DTel of the mutant (Fig. 4.10 f). At 15.5 Pancytokeratin expression is restricted to the choroid plexus both in the control (Fig. 4.10 c, d) and the mutant (Fig. 4.10 g) telencephalons. The mutant does not show any additional expression of Pancytokeratin in the DTel (Fig. 4.10 h).

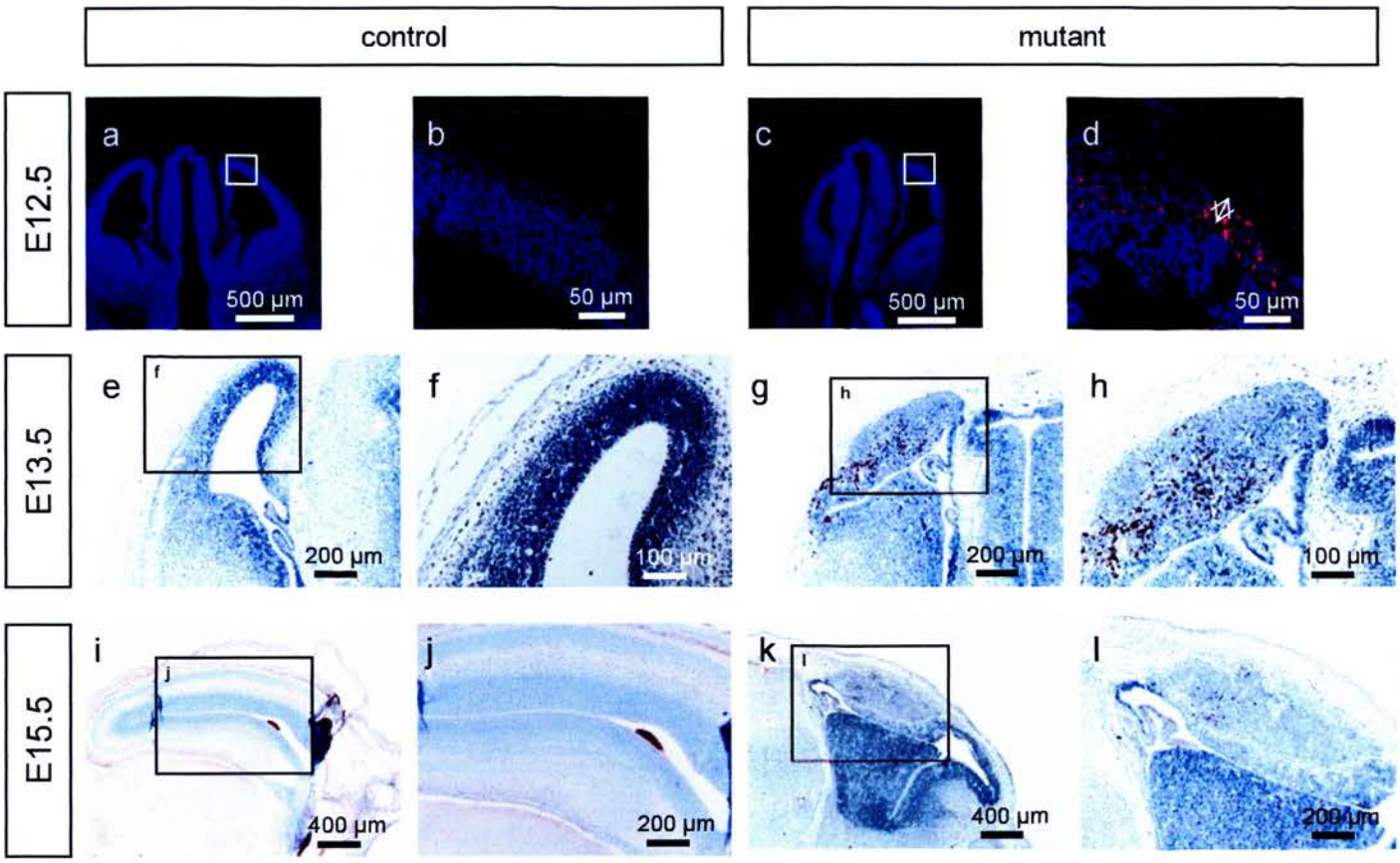


Fig. 4.8 . **WT1 expression in the telencephalon of control(*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5, E13.5, and E15.5.** There is no expression of WT1 in the control telencephalon at E12.5 (a, b) and E13.5 (e, f). At E15.5 there is WT1 staining in the cell processes (i, j). At E12.5 the mutant dorsal telencephalon has WT1 positive cells in upper layers (red, d, white arrows). At E13.5 cells positive for nuclear WT1 spread in the dorsal telencephalon (g) with higher expression in the middle (h). A sagittal section at E15.5 shows more cells positive for nuclear WT1 in the caudal area of the dorsal telencephalon (k, l). Wt1 – red (a-d), brown (e-l). Blue –ToPro-3 nuclear counterstaining (a-d), haematoxylin (e-l).

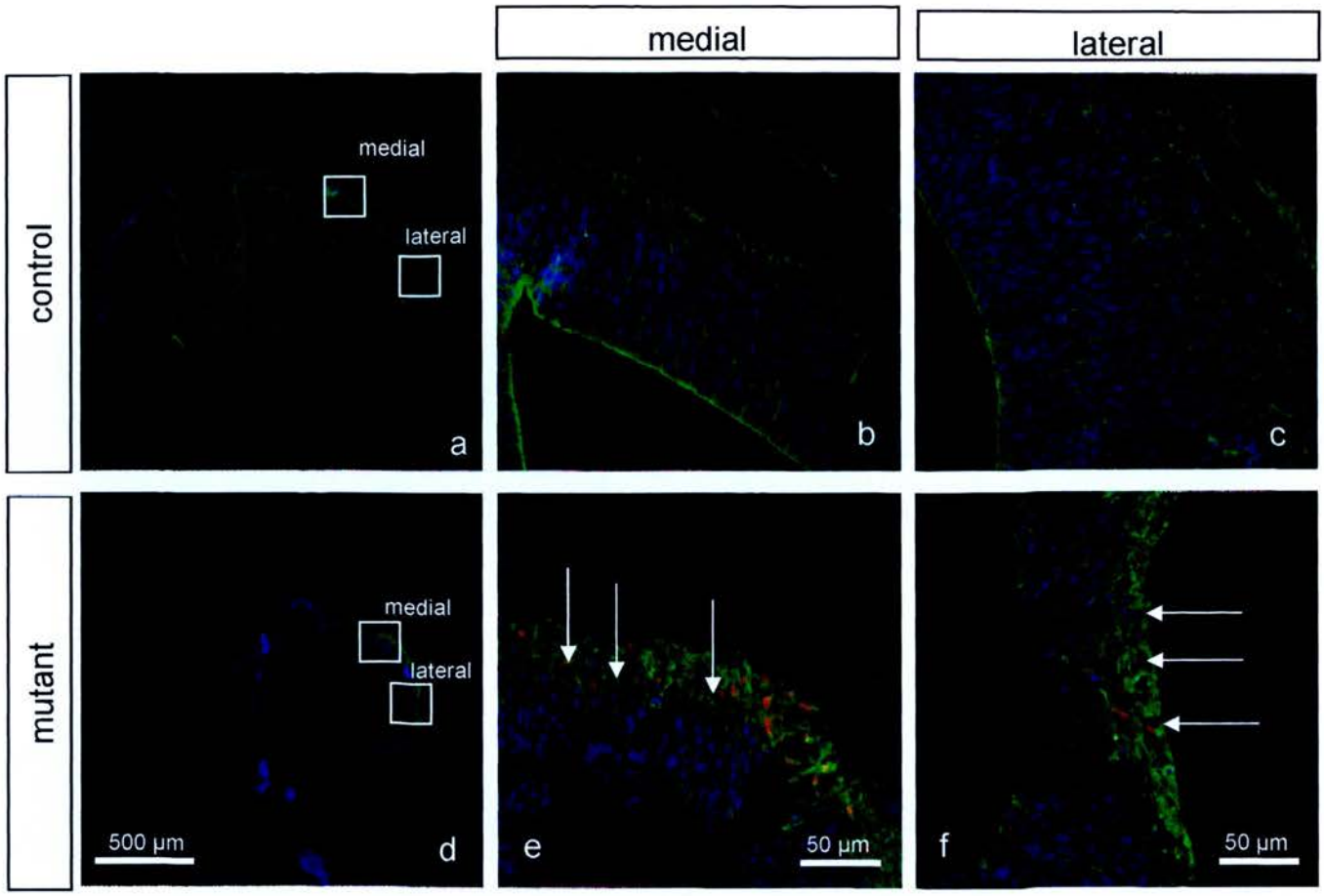


Fig. 4.9 . **Double immunostaining for Wt1(red) and beta-catenin (green) in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5**
There is no expression of Wt1 in the control (b and c). There is no nuclear beta-catenin in the control (b and c). Nuclear beta-catenin and Wt1 expression is overlapping in the mutant (e and f, white arrows). Blue –ToPro-3 nuclear counterstaining.

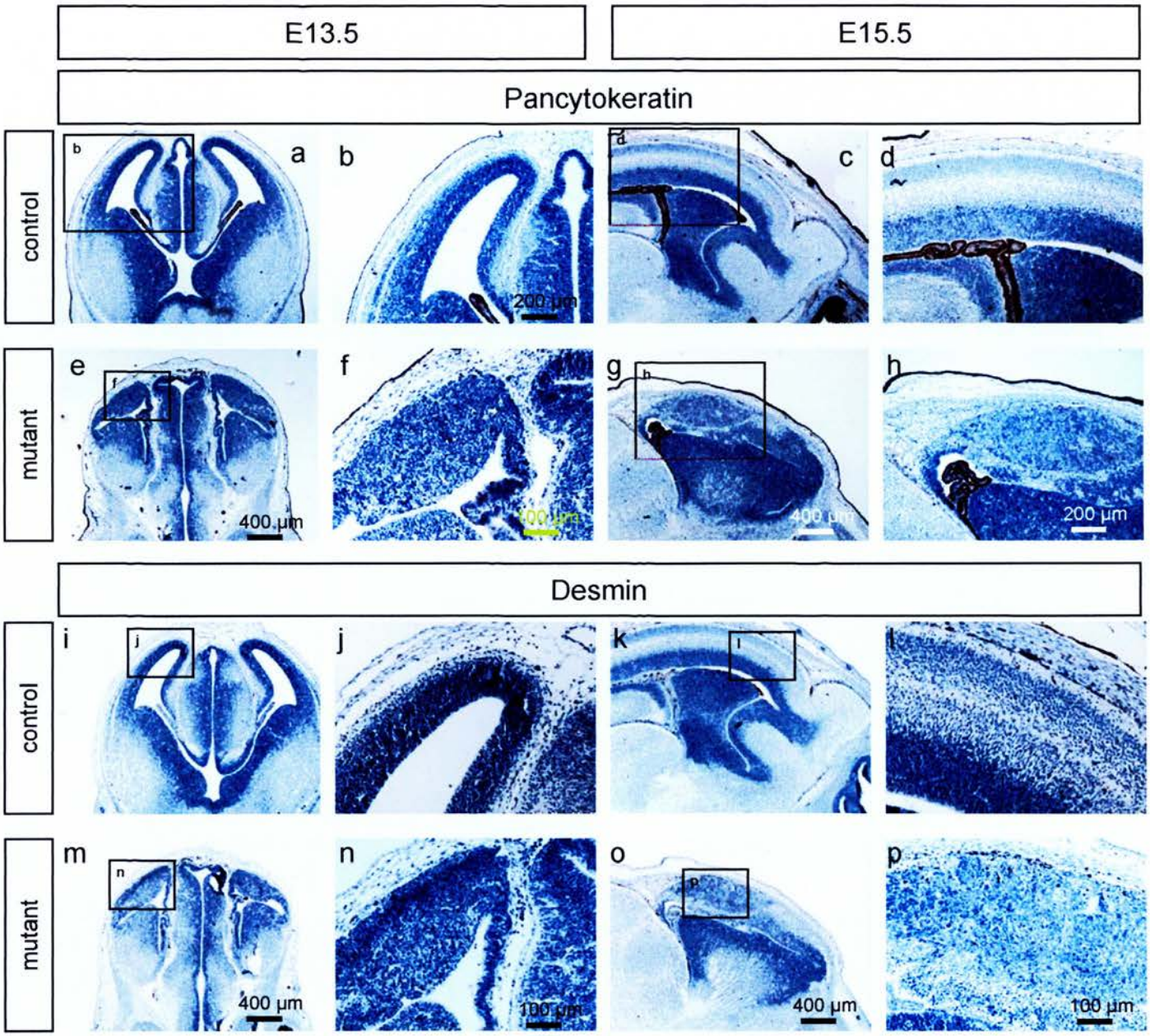


Fig. 4.10. **Pancytokeratin (brown) (PCK) and desmin (brown) expression in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 and E15.5.** Pancytokeratin expression is restricted by choroid plexus at E13.5(a,b) and E15.5(c, d). Mutant PCK-positive cells are also restricted to the choroid plexus: E13.5 (e, f) and E15.5 (g, h). There is no presence of desmin in the dorsal telencephalon of the control and the mutant at E13.5(i, j and m, n, respectively) and at E15.5 (k, l and o, p, respectively). Blue – counterstaining with haematoxylin.

Desmin is an intermediate filament. Desmin is one of the first detected muscle proteins in the heart and somites (Li et al., 1993). Desmin is also expressed by smooth muscles. There is no expression of desmin by the DTels of the control (Fig. 4.11 i, j) and the mutant (Fig. 4.11 m, n). Desmin is not expressed in the DTel at E15.5 by control (Fig. 4.11 k, l) and mutant (Fig. 4.11 o, p).

S100 is a family of 21 Ca-binding proteins. The level of S100 is elevated in malignant melanomas. S100 proteins are expressed in astrocytes, oligodendrocytes, Schwann cells, adrenal medulla and a variety of other cells including chondrocytes, adipocytes, and melanocytes (Harpio and Einarsson, 2004). There is no detectable expression of S100 in the control (Fig. 4.11 a, b) and mutant (Fig. 4.11 e, f) DTels at E13.5. At the later stage (E15.5) the DTel of the control and the mutant remains negative for S100 (Fig. 4.11 c, d and g, h, respectively).

MyoD1 is a skeletal muscle specific protein. MyoD1 has a role in myogenic determination and differentiation (Sassoon et al., 1989). MyoD1 is a rhabdomyosarcoma marker. The control DTel does not express MyoD1 at E13.5 (Fig. 4.11 i, j) and E15.5 (Fig. 4.11 k, l). Cells of the mutant DTel remain negative for MyoD1 at E13.5 and E15.5 (Fig. 4.11 m, n and o, p, respectively).

Vimentin is a type III intermediate filament. Higher expression of vimentin correlates with higher invasive abilities of a tumour (Ngan et al, 2007). Vimentin can be used as a marker of oncogenic progression (Kryszke and Vicart, 1998). The transcription

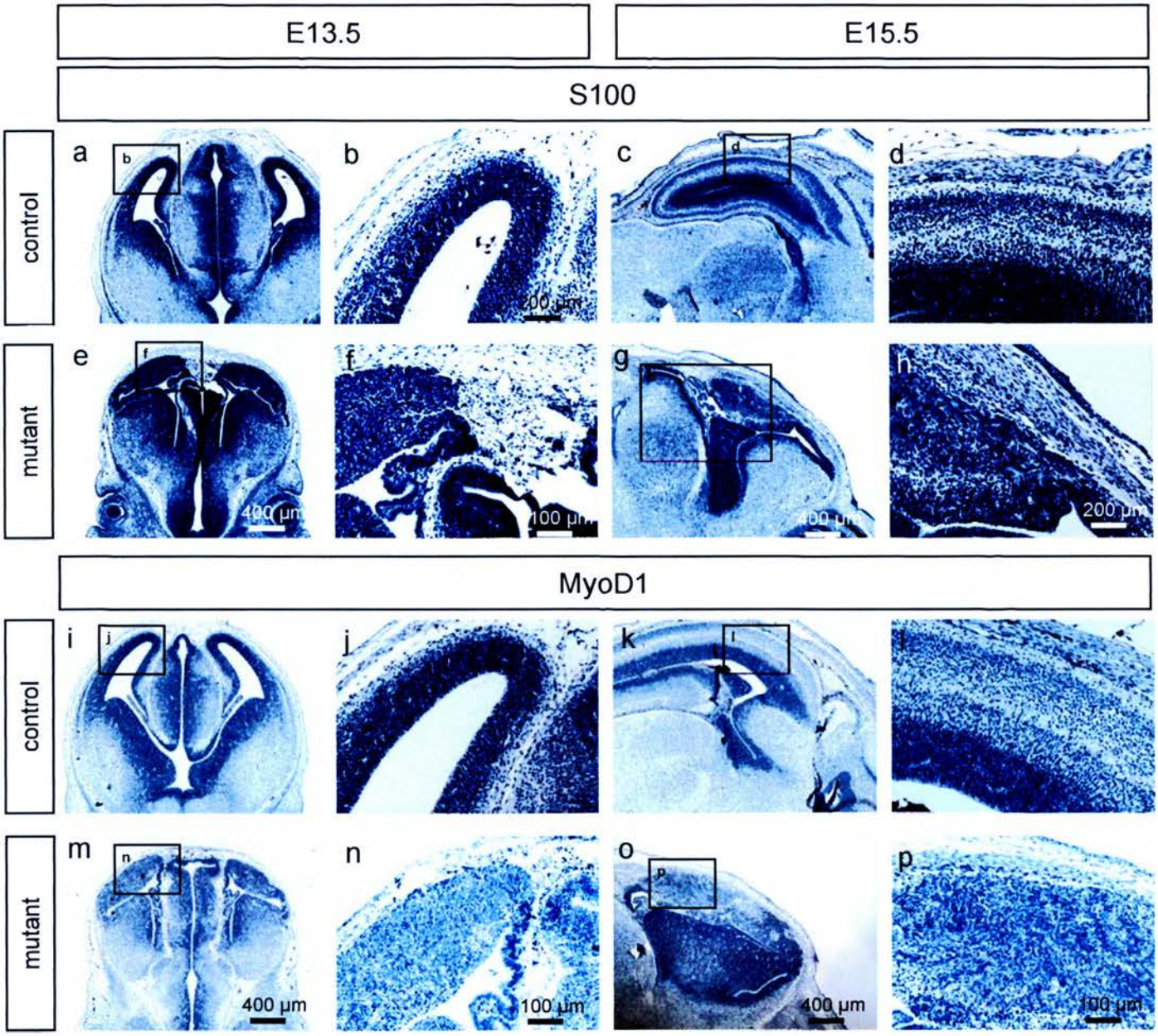


Fig. 4.11. **S100 (brown) and MyoD1 (brown) expression in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 and E15.5.** Both S100(a-h) and MyoD1(i-p) expression is absent in the dorsal telencephalon of the control and the mutant at E13.5(a,b,e,f,I,j,m,n). and E15.5(c,d,g,h,k,l,o,p). Blue – counterstaining with haematoxylin

factor PEA3, encoded by a member of the ets oncogene family, activates the vimentin promoter in mammary tumor cells (Kryszke and Vicart 1998). Vimentin is not expressed by either the control or mutant DTels at either E13.5 (Fig. 4.12 a, b and e, f, respectively) or E15.5 (Fig. 4.12 c, d and g, h, respectively).

Smooth muscle actin (SMA) is a myoepithelial marker. The control and the mutant DTels show no detectable level of SMA at E13.5 in the DTel (Fig. 4.12 i, j and m, n, respectively). However surrounding tissue (connective tissue – future skull) expresses SMA in the control (Fig. 4.12 j) and the mutant (Fig. 4.12 n). A similar pattern of SMA expression is found at E15.5 in the control (Fig. 4.12 k, l) and the mutant (Fig. 4.12 o, p).

Taken together, the findings of my marker analysis indicate that cells of the mutant telencephalon do not change their neuronal identity. Further, and surprisingly, WT1 expression is up-regulated in mutant cells.

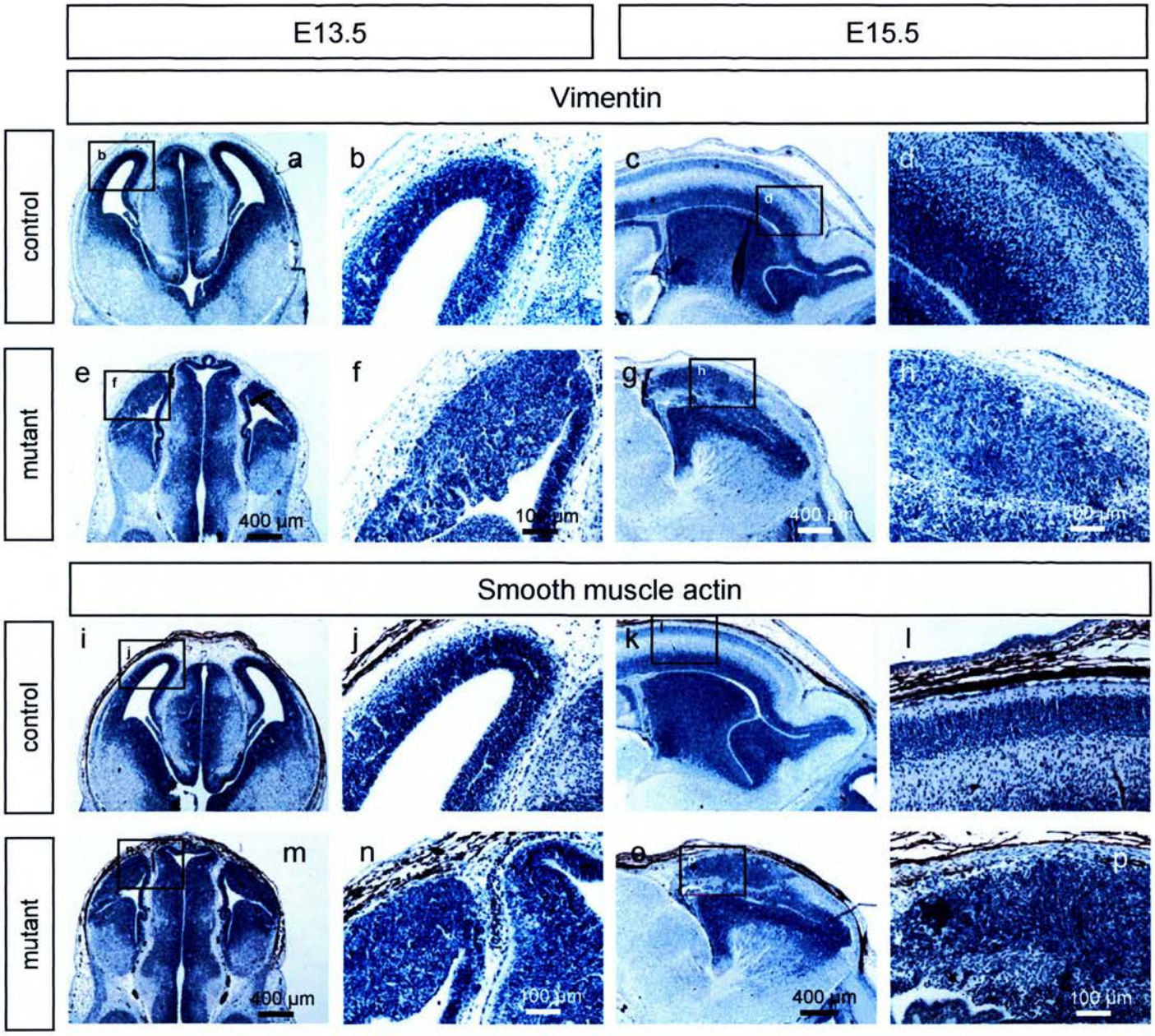


Fig. 4.12 . **Vimentin (brown) and smooth muscle actin (SMA) (brown) expression in control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 and E15.5.** There are no positive cells for vimentin (a-h) and SMA (i-p) in the dorsal telencephalon of the control and the mutant at E13.5(a,b,e,f,I,j,m,n). and E15.5(c,d,g,h,k,l,o,p). Blue – counterstaining with haematoxylin

4.7 Discussion

4.7.1 Foxg1

A hypothesis is that Apc mutation, leading to activation of Wnt signalling through stabilization of beta-catenin, may lead to an increase in the size of regions which require Wnt signalling for their development. There is evidence in zebrafish that local antagonism of Wnt signalling is required to establish the telencephalon from anterior ectoderm (Houart et al., 2002). Heisenberg et al. (2001) showed that changes in an amino acid sequence of Axin1, a protein of the beta-catenin destruction complex, caused *masterblind* mutation in zebrafish, where diencephalic fates expand rostrally, causing reduction or absence of the telencephalon and eyes. Studies carried out on chick embryos (Gunhaga et al., 2003) showed that Wnt signalling is important for development of the telencephalic character of the neural plate. Cre expression depends on *Emx1* expression, therefore *Apc* is mutated when the cerebral cortex is specified. An additional chicken brain development study provided information that Wnt signalling is important for acquiring caudal telencephalic, midbrain, and hindbrain properties (Nordstrom et al., 2002). Backman et al. (2005) showed in a mouse model that increased beta-catenin activity in the subpallium led to expansion of the dorsal telencephalic fate ventrally. There is an opposite effect if beta-catenin activity is decreased.

Data in this chapter shows that the mutant dorsal telencephalon loses Foxg1 expression from E12.5. There are only single cells which express Foxg1 later in development. One explanation for this may be that Wnt signalling over-activation causes

an acquisition of more caudal fates than telencephalic fates in the dorsal telencephalon. Heisenberg et al., (2001) presented data that *mbl*$^{-/-}$ embryos, which lack Axin1, were affected from gastrulation and showed reduction in expression of telencephalic genes such as *emx1* whereas expression of mid/caudal diencephalic genes expanded rostrally. Houart et al. (2002) investigated this topic in more detail. They presented data that lost of telencephalic tissue in *mbl*$^{-/-}$ embryos is due to over activation of Wnt signalling, namely Wnt8b, and telencephalic fates in masterblind embryos can be restored if Wnt8b activity is abrogated (Houart et al., 2002). Also there is a possibility that the boundary between the telencephalon and diencephalon is compromised, leading to mixing of cells of more caudal regions (diencephalon) with rostrally (telencephalon) derived cells. However, the morphological boundary is still present in the mutant forebrain and Foxg1 negative tissue is mainly Apc negative also, which suggests that probably these cells have lost Foxg1 expression because of *Apc* mutation. Additional possibility is that Foxg1 negative tissue of the dorsal telencephalon reflects enlargement of the cortical hem. However these two latter hypotheses were not tested in details.

4.7.2 Pax3

Pax3 is expressed in the developing nervous system in the dorsal part of the neural tube, the mesencephalon, and the neural crest derivatives normally (Tremblay et al., 1995). Later was shown that Pax3 participates in early neuroaxis patterning in Xenopus (Bang et al., 1999). Data presented by Bang et al. (1999) suggest that Wnt signalling is important for induction of Pax3 expression as induction of Pax3 is blocked by a dominant-negative form of XWnt-8. Work on a mouse model done by Monsoro-

Burq et al. (2005) indicated that induction of the neural crest required induction of Pax3 through the canonical Wnt pathway. Matsunaga et al. (2001) carried out misexpression studies which showed that Pax3 with Pax7 expressed ectopically in the diencephalon could change diencephalon identities to the mesencephalic tectum. These works express a possibility of Wnt signalling to induce Pax3 expression, which, in turn, can shift identities in a region of misexpression. My data show that there are many Pax3 expressing cells in the mutant dorsal telencephalon at E15.5. However, Pax3 expression at E12.5 is detected in a few cells in the mutant DTel. But Foxg1 expression is absent in most of the mutant DTel at E12.5, which suggests that telencephalic identity of the mutant cortex was lost before Pax3 expression. This means that the caudal to rostral shift appears later than E12.5 in brain development, suggesting that ectopias of Pax3 expression reflect an induction of Pax3 expression after the mutant DTel lost its telencephalic identity rather that stimulated ectopic Pax3 expression causes loss of the telencephalic identity of the mut DTel. Increased expression in the mutant DTel of *Wnt8b* from E13.5, but a normal level at E12.5, suggests a similar explanation. Thus, a possible sequence of changes is that the mutant DTel loses its identity first, and then there is a start of expression of caudal genes.

4.7.3 The roof plate

The roof plate organizes development of the dorsal and medial pallium, including the hippocampus and choroid plexus (Mack et al., 2001). Pancytokeratin (a

choroid plexus marker) staining does not expand into the dorsal telencephalon, suggesting that there is no expansion of roof plate structures. Therefore it is possible to assume that there is no middle to medial shift in the mutant DTel. However further investigation is needed to justify this assumption.

4.7.4 Pax6, Tbr2, and Tbr1

Activation of Wnt signalling by beta-catenin stabilization leads to an expansion of dorsal telencephalic regions ventrally (Backman et at., 2005). Data in this chapter shows that the dorsal telencephalic marker Pax6 has no additional expression in the ventral telencephalon. It is possible to conclude that dorsal activation of Wnt signalling in the mutant telencephalon does not spread ventrally. Moreover expression of Pax6 is decreased in the dorsal part of the mutant telencephalon. Pax6 has an essential role in radial glia differentiation (Gotz et al., 1998). As was shown by Englund et al. (2005) apical progenitor cells express Pax6. It is possible to suggest that decreased Pax6 expression causes a decreased population of intermediate progenitor cells and post-mitotic neurons. Indeed, Tbr2, a basal progenitor cell marker (Englund et al., 2005), has decreased expression in the mutant. Early post-mitotic neuronal marker Tbr1 (Englund et al., 2005) expression is decreased also. Pax6, Tbr2, and Tbr1 reflect sequential differentiation of the radial glia cells into neurons (Englund et al., 2005). Populations of cells expressing particular differentiation markers occupy particular regions in the cortex (Englund et al., 2005). Expression of all three markers shows that all stages of neuronal maturation are present in the mutant DTel. It is possible that the presence of Pax6, Tbr2,

and Tbr1 positive cells might correspond to cells which nave not deleted *Apc* (see chapter 3). However these three populations localize in same areas of the telencephalic tissue. Also undifferentiated mutant tissue compresses marker expressing cells into *VZ area. Incorrect localization of these cells in the mutant DTel may be due one or more of the following reasons: defects of polarity, defects of cell migration, and defects in guiding cues. The Pax6 decrease may correspond to a decrease of the apical progenitor pool, whereas the Tbr2 decrease reflects a decreased basal progenitor population, and the Tbr2 decrease shows a reduced population of post-mitotic neurons. If all stages of neuron maturation are diminished it is possible to assume that progenitor cells proliferate at similar rates.

4.7.5 Tangential migration

One of Pax6 functions is regulation of tangential migration in the telencephalon through PSB maintenance (Chapouton et al., 1999). Therefore there is a possibility that the PSB is compromised because Pax6 expression is decreased in this region. Stoykova and colleagues (1996) showed that Sey mutation of Pax6 led to disruption of the cortical-striatal boundary. As evidence there are more cells migrating from the subpallium into the cortex when Pax6 is mutated (Stoykova et al., 2000). Indeed, the mutants exhibit the presence of cells positive for a ventral telencephalic marker Olig2 in the mutant DTel. This can suggest increased permeability of the PSB. However there are no ventral to dorsal expansions of expression of ventral telencephalic markers Mash1 or Islet1. Expression of these two genes remains similar to that in the control

telencephalon. According to the latter fact it is possible to assume that PSB function is not severely affected. The explanation of Olig2, but not Mash1 and Islet1, presence in the mutant DTel could be the following. There are additional roles of Olig2 in the brain besides expression in oligodendrocytes and formation of the motoneuron progenitor pool (Mizuguchi et al., 2001). An important function of Olig2 is participation in brain injury response (Buffo et al., 2005). Olig2 expression in the nervous system is up-regulated in response to ischemia, stab lesions, demyelination, or immune mediated injury (Fancy et al., 2004). Possibly Olig2 positive cells in the mutant DTel reflect a response to injury/developmental defects.

4.7.6 Neurogenesis

Ngn2 is a neuronal determination gene essential for neurogenesis (Parras et al., 2002). Decreased expression of neuronal maturation markers Pax6, Tbr2 and Tbr1 in the mutant dorsal telencephalon can lead to a hypothesis that Ngn2 expression decreased also, because of Ngn2 participation in the neurogenesis. Also previous studies of Scardigli et al. (2003) showed that Ngn2 was positively regulated by Pax6 expression in the pallium. However there is more expression of Ngn2 in the mutant compared to the control. Backman et al. (2005) published data that beta-catenin is required for maintenance of expression of dorsal telencephalic markers Emx1, Emx2, and Ngn2. If beta-catenin is inactivated then expression of these markers is down-regulated (Backman et al., 2005). A possible explanation of increased Ngn2 expression is that stabilized beta-catenin has an opposite effect on Ngn2 distribution than inactivated beta-catenin. In

support of this hypothesis there are results of Israsena et al. (2004), which show that Ngn2 expression is activated by beta-catenin signalling. However these experiments were carried out on neural stem cells. Additionally, in slice culture experiments Miyata et al (2004) found that the concentration of Ngn2 depends on the cell cycle phase – the highest concentration of Ngn2 is in G1 phase. These data provide additional explanations of broadened Ngn2 expression and is in good correlation with cell-cycle investigation results described in Chapter 5. However G1-arrest of the mutant cells is probably a secondary affect in the sequence of events. In other words cells are blocked in G1-phase and therefore have an increased level of Ngn2. Also Ngn2 is expressed at the ventral midline of the midbrain from E10.5 (Andersson et al., 2007). As there is a possibility of a caudal to rostral shift (Pax3, Wnt8b Wnt1 expression in the dorsal telencephalon), increased Ngn2 expression may reflect a caudal to rostral change as well. Also Helms with colleagues (2005) published data that Ngn2 and Mash1 have overlapping expression in the spinal cord. Also they showed that Ngn2 functions down-stream of Mash1 in the generation of spinal cord interneurons. However there is no Mash1 expression in the mutant DTel, which makes this assumption very weak. Thus, increased Ngn2 expression may reflect different processes in the mutant tissue, but there is a high probability that this is due to G1 cell cycle arrest after stabilization of beta-catenin.

4.7.7 Wt1

Wt1 was described as an important factor in kidney development. Wt1 is responsible for mesenchyme to epithelium transition in renal development (Perantoni et al., 1995). Wt1 is expressed in tissue of ectodermal origin: spinal cord and brain (Armstrong et al., 1993). An indispensable role of Wt1 in olfactory system development was described by Wagner et al. (2005). Moore et at. (1999) presented data that Wt1 is responsible for forward and back changes between mesenchymal and epithelial states. Also Wt1 has a role in tumour development. However its function in tumour development is not clear. One study shows that Wt1 has tumour suppressor properties by stimulating p53-independent apoptosis and it is mutated in subset of tumours (Haber et al., 1996) whereas others show that Wt1 is up-regulated in certain tumours (Sugiyama 2001) and its suppression leads to inhibition of tumour cell growth (Oji et al., 1999). According to Dennis et al (2002), though Wt1 expression is found in gliomas, neuroblastomas, and medulloblastomas, Wt1 does not have a major role in the aetiology of neural tumours. My data shows that Wt1 is present in neuronal processes in the control DTel. Up-regulation of Wt1 expression from E12.5 in the mutant DTel may reflect several possibilities: changes in tissue identity, tumour development, and tumour suppression. However its function in the brain is unclear.

The first probability is changes of ectodermal (neuronal) identity to the mesodermal identity. However desmin and MyoD1 which mark tissues of mesodermal origin reveal no positive staining in the mutant DTel. Therefore it is possible to suggest that epithelial-to-mesenchymal transition is not the cause.

The second possibility is tumour development in the DTel of the mutant. The panel of antibodies to different types of tumours did not reveal positive results, except from Wt1. This up-regulation of Wt1 can show changes related to a tumour formation but the mutant tissue is negative for other tumour growth markers such as Vimentin, MyoD1, and S100. Also cell cycle analysis (described in detail in chapter 5) found decreased proliferation in the mutant tissue. Thus Wt1 up-regulation as a tumour development marker does not look strong but might reflect suppression of tumour formation at the early stage.

The third option is up-regulation of Wt1 as a reflection of tumour suppression. The tumour suppression function of Wt1 is related to apoptosis induction. My data (described in detail in chapter 5) show that apoptosis is increased in a p53-independent way. Also data in chapter 3 show p21 up-regulation which also fits with this hypothesis, as Englert et al. (1997) describe the possibility of p21 induction by Wt1 independently of p53. In support of this assumption is decreased expression of Wt1 in the mutant DTel at E15.5 compare to E13.5, which might reflect apoptosis of cells with a high level of Wt1.

Thus, my data are more consistent with the hypothesis of Wt1 up-regulation as a reflection of tumour suppression.

An additional question is a mechanism of Wt1 up-regulation in the developing mutant tissue. As was shown in chapter 3 after Apc deletion there was a beta-catenin nuclear translocation with activation of Wnt target genes. Also there are number of works which show nuclear beta-catenin translocation in tumours (McEntee et al., 1999; Iwamoto et al., 2000). Koesters with colleagues (2003) presented results which show

nuclear translocation of beta-catenin to the nucleus in Wt1 related tumours. In addition to this Amini et al. (2005) showed Wt1 is over-expressed in tumour with stabilized beta-catenin. There is a link between Wnt activity and Wt1 expression. Gene profiling experiment done by Discenza and co-workers (2004) identified *Wt1* gene downstream of *Pea3* and Pea3 promotes expression of *Wt1*. Previous studies by a different research group found that Pea3 is highly expressed in tumours from *Wnt1* expressing mice (Howe et al., 2001). As was shown in Chapter 3 the mutant DTel has increased expression of Wnt genes - *Wnt1* and *Wnt8*. Taken together these facts fill in gaps in the connection between Wnt signalling and Wt1. Thus, a current hypothesis of Wt1 up-regulation in the mutant DTel is that after Apc deletion there is stabilisation of beta-catenin which leads to up-regulation of Wnts (Wnt1 in particular); this in turn leads to Pea3 activation with following enhancement of Wt1 expression.

4.8 Conclusion

In conclusion, the mutant demonstrates altered expression patterns of different genes in the DTel. There are strong signs of caudalization of the DTel with decreased expression of the dorsal telencephalic markers. There is evidence that these changes are mostly because of stabilization of beta-catenin with followed by activation of the canonical Wnt pathway.

5. APC: effects on proliferation, cell death, and ploidy

5.1 Introduction

Cell division, cell maturation, and cell death must be tightly regulated during morphogenesis in order to generate a structure with the proper size and shape.

The Apc^{580S} mutant develops a smaller DTel than the control does. This could be due to increased cell cycle length, increased cell death, decreased proliferative pool or their combination.

Wnt signalling might be considered as a candidate mechanism underlying the effects of loss of *Apc*, because Apc is involved in its regulation. One of the roles of Wnt signalling is regulation of proliferation during development (Moon et al., 1997). Bradley and Brown (1995) showed that medium containing Wnt1 induced mitogenesis in a mammary cell line. Beta-catenin is a critical component of the canonical Wnt signalling pathway (Fagotto et al., 1998). Its stabilization leads to an increase of the number of proliferating cells without an increase of the cell cycle rate in the developing mouse cortex (Chenn and Walsh, 2002). Increase of the progenitor pool can be due to more cells choosing to proliferate instead of differentiating (Chenn and Walsh, 2002). Activation of Wnt signalling can have various effects on proliferation and neuronal differentiation. It was shown that absence of Wnt1 and Wnt3a produces a deficiency of the neural crest derivatives and a drastic reduction of the neural precursor pool (Ikeya et al., 1997). Megason and McMahon (2002) showed the mitogenic activity of Wnt1 and

Wnt3a in developing spinal cord. Stimulation of neurogenic progenitor proliferation by Wnt7a and Wnt7b was described by Viti et al. (2003). Hirabayashi et al. (2004) presented data which showed that Wnt7a or stabilized beta-catenin promote neuronal differentiation of neural precursor cells in mouse cortical cultures. Also, this research group showed that inhibition of the canonical Wnt pathway suppresses neuronal differentiation in the developing cerebral cortex. These different effects of Wnt signalling stimulation can be explained by a hypothesis that Wnt signalling activation can have different effects at different stages (Hirabayashi et al., 2004). Indeed experiments done by this group revealed that neuronal differentiation during cerebral cortex development was induced by canonical Wnt signalling at E13.5 but not at E10.5.

There is considerable evidence that mutation of *Apc* is important for development of polyps in the colon (Nagase and Nakamura, 1993; Smith et al., 1993). There is evidence that APC is mutated in around 80% of colorectal tumours (Miyoshi et al., 1992). Rescue experiments with induction of expression of full-length Apc in colorectal cancer cells which lack Apc showed that cell growth decreased due to induction of apoptosis (Morin et al., 1996). However Sansom et al. (2004) reported an increased level of apoptosis in the colon crypt using Apc^{580S} and the Cre-loxP system to knock-out Apc. Hasegava and colleagues (2002) carried out experiments disrupting *Apc* function in the neural crest specifically using the Cre-loxP system also. They found that apoptosis was increased in the neural crest causing severe developmental defects. A possible explanation of this discrepancy in Apc influence on apoptosis was presented by Venesio et al. (2003), who showed consistent reduction of apoptosis in cases where *Apc* had a frameshift mutation in codon 1383. Other mutations did not show similar effects

on cell death (Venesio et al., 2003). Thus, it is possible to suggest that different mutations in Apc have different effects on apoptosis.

Many cancers have chromosomal instability, resulting in gain or loss of chromosomes or their parts (Lengauer et al., 1998). It has been shown that Apc mutant cells had defective mitotic spindle formation leading to aberrant chromosomal segregation (Fodde et al., 2001). Supporting results were received in experiments with expression of truncated Apc forms in cultured cells, which promoted chromosomal instability leading to high levels of aneuploidy (Tighe et al., 2004). Dikovskaya et al. (2007) provided evidence that loss of Apc led to polyploidy as a consequence of disregulation of mitosis and apoptosis. Therefore it is possible that euploidy of cells in the DTel might be affected in the $Emx1^{Cre/+}Apc^{580S/580S}$ embryos.

5.2 Proliferation

Proliferation in the DTel may be altered when Apc is mutated. Experiments of Hirabayashi and co-workers (2004) suggest that early activation of Wnt signalling by stabilizing beta-catenin does not stimulate neurogenesis. Chenn and Walsh (2002) found increased proliferation in telencephalon of transgenic embryos that express stabilized beta-catenin after E10.5. In my model deletion of *Apc* occurs after E9.5 as the *Emx1* promoter is used to drive Cre-recombinase expression. Nuclear localized beta-catenin appears at E10.5 (see details in chapter 3) demonstrating that it is stabilized at least in some cells shortly after Cre activation. As was shown in a previous chapter, neuron

specific staining (TuJ1 – chapter 3, Fig 3.10) at E13.5 shows that TuJ1 is more abundant in the mutant DTel compared to the control one. Also staining with the progenitor marker Nestin at E13.5 (chapter 3, Fig 3.9) reveals decreased expression in the DTel of the mutant compared to the control. Thus, marker staining suggests a decrease in the proliferative pool and an increase in neural differentiation. However the time of *Apc* mutation supports a suggestion of an increase in proliferation as lost of *Apc* leads to stabilization of beta-catenin, which drives neural precursor cell proliferation at early stages.

To determine when loss of Apc affects proliferation in DTel, I investigated cell cycle parameters in mutant embryos

Proliferating cell nuclear antigen (PCNA) is an important protein in DNA replication and repair (Gary et al., 1997). Expression of PCNA marks proliferating cells (Baserga 1991). Deletion of *Apc* might have regional specificities and, therefore, the dorsal telencephalon was divided into three regions: caudal, middle, and rostral, which in turn were subdivided into medial and lateral areas. $Emx1^{Cre/+}APC^{580S/580S}$ (mutant) animals show decreased PCNA expression compared to the $Emx1^{Cre/+}APC^{580S/+}$ (control) animals at E12.5 and E13.5 (Fig 5.1 a-f and Fig 5.2 a-f, respectively). At E12.5 quantification of PCNA expression in different regions of the DTel (medial and lateral areas in caudal, middle and rostral regions) revealed a significant decrease in the number of PCNA expressing cells in the caudal-medial and middle-medial regions of the mutant DTel compared to respective zones of the control (Fig 5.1.g). Similar quantification was carried out at E13.5. At this age a significant decrease in the number of PCNA

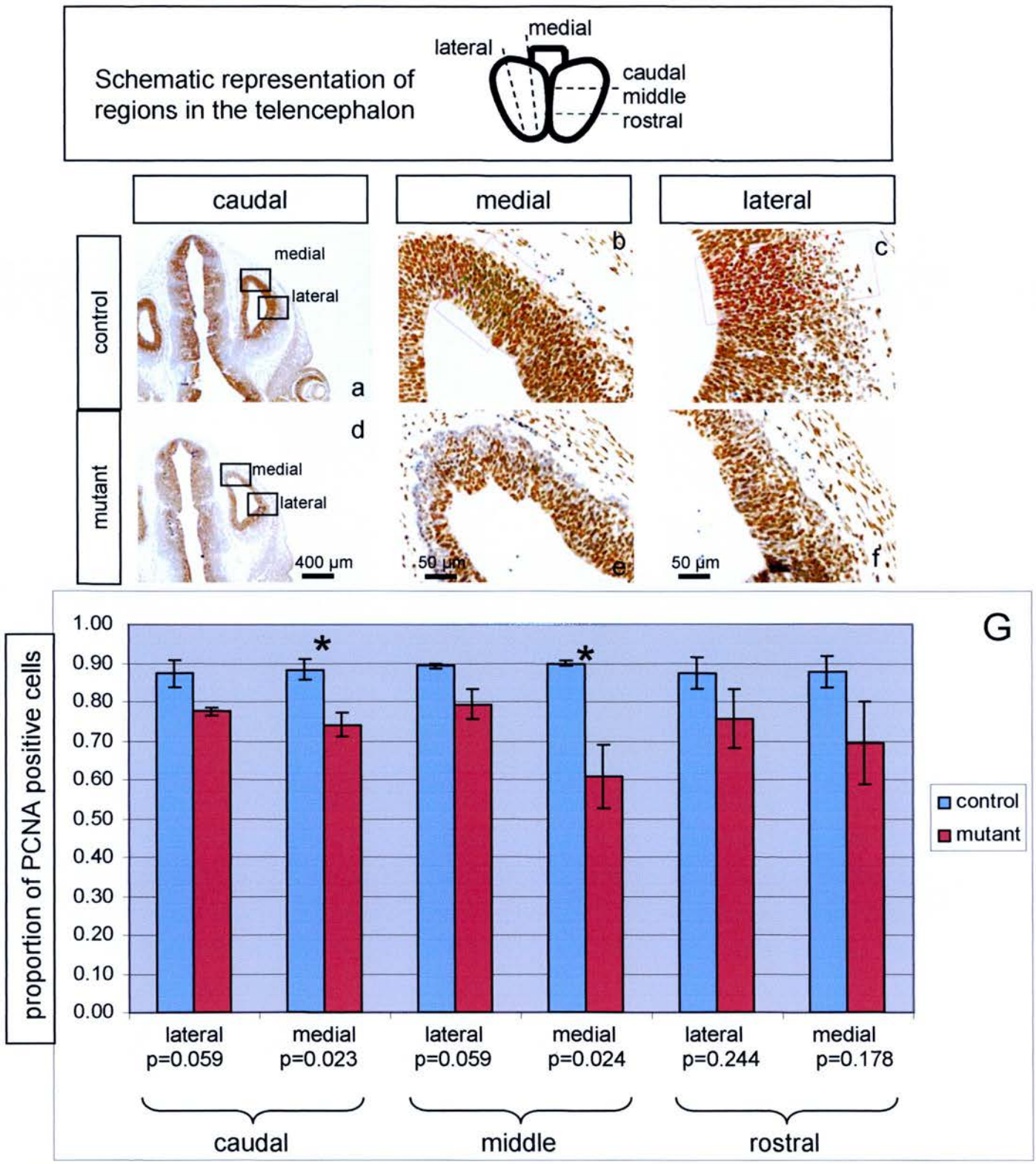


Fig 5.1 PCNA (brown) staining and quantification of PCNA-positive cells in the DTel of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5. Immunostaining shows that PCNA is expressed in the control (a, b, c) and the mutant (d, e, f). Quantification of PCNA positive cell number expressed as proportion of all cells (g) shows that PCNA expression is significantly decreased in the mutant DTel in caudal-medial and middle-medial regions. T-test performed. N= 3 embryos. Error bars are SEM.*-significant difference. Blue staining – haematoxylin.

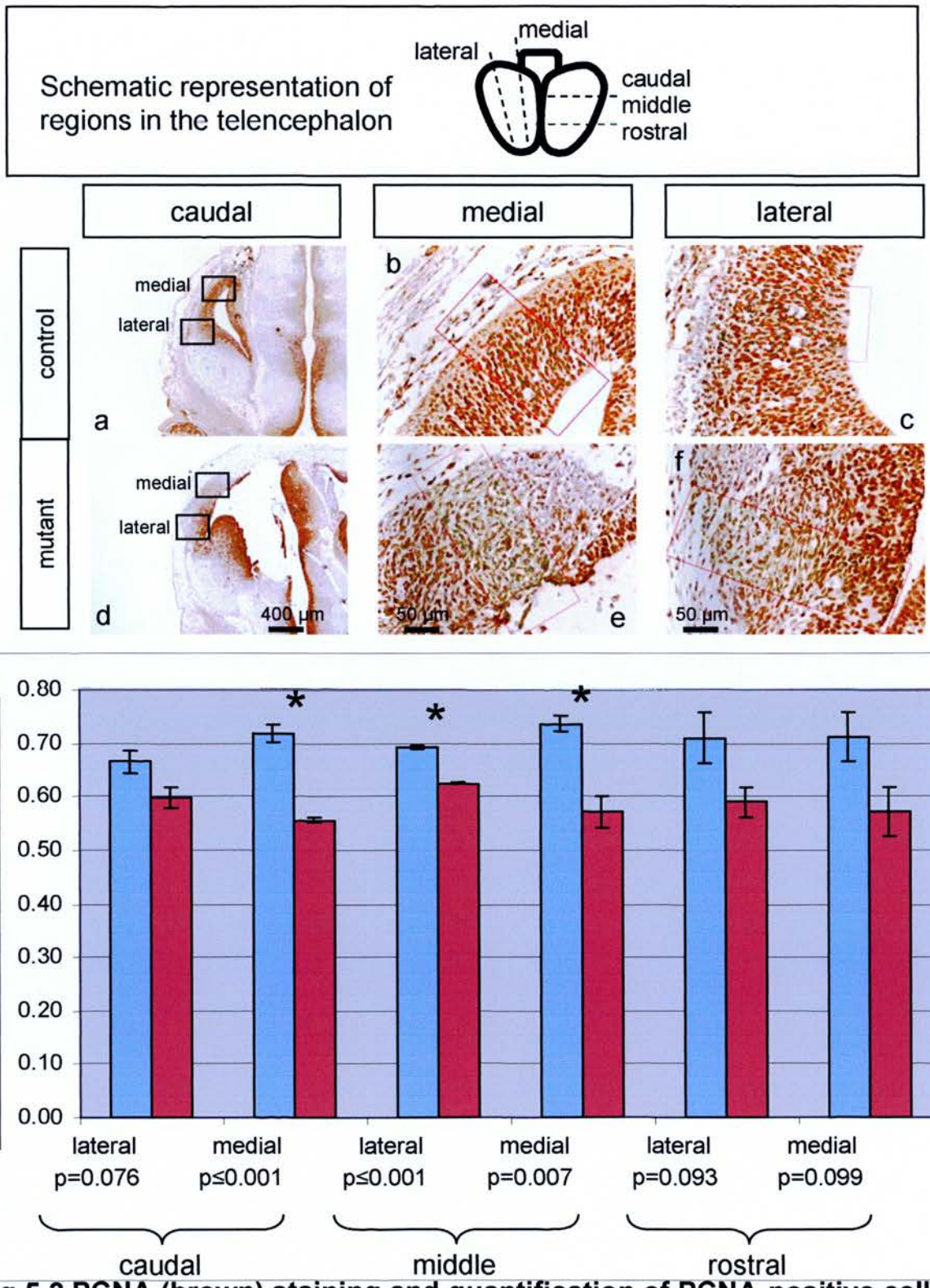


Fig 5.2 PCNA (brown) staining and quantification of PCNA-positive cells in the DTel of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 Immunostaining shows that PCNA is expressed in the control (a, b, c) and the mutant (d, e, f). Quantification of PCNA positive cell number expressed as proportion of all cells (g) shows that PCNA expression is significantly decreased in the mutant DTel in caudal-medial, middle-medial, and middle lateral regions. T-test performed. N=3 embryos. Error bars are SEM. *-significant difference. Blue staining – haematoxylin

expressing cell was found in the caudal medial, middle-medial, and middle lateral regions (Fig 5.2 g).

An alternative way to label dividing cells is to use the nucleotide analogue BrdU, which is incorporated by cells undergoing S-phase (Coltrera and Gown, 1991). Continuous cumulative BrdU injection labels virtually all dividing cells (Nowakowski et al., 1989). Immunostaining of the DTels of embryos continuously labeled with BrdU for 12 hours revealed that the DTel of the control and the mutant have different proportion of cells which incorporated BrdU at E12.5 (Fig 5.3 a-f) and E13.5(Fig 5.4 a-f). Quantification of the relative size of the BrdU-incorporating population disclosed a significant reduction of BrdU positive cells in the middle-medial region of DTel in the mutant compared to the equivalent area of the control (Fig. 5.3 g). At E13.5 there are two regions in the mutant DTel which exhibit significant reduction of BrdU incorporating cells compare to the control: middle-medial and middle rostral (Fig 5.4 g).

Data from PCNA expression and continuous BrdU labeling show similar tendencies for a decrease of the proliferative pool in the DTel. Also more severe defects in proliferation are found in medial areas of the DTel. This might reflect a gradient in expression of *Emx1* in the DTel (high medial, low lateral). Apc immunostaining reveals Apc negative regions medially but not laterally in the mutant DTel (Fig 3.3). However medial-lateral difference in Wnt signaling disregulation was not quantified in the current work. One way to do it would be a comparison of the percentage of nuclear beta-catenin positive cells located medially and laterally.

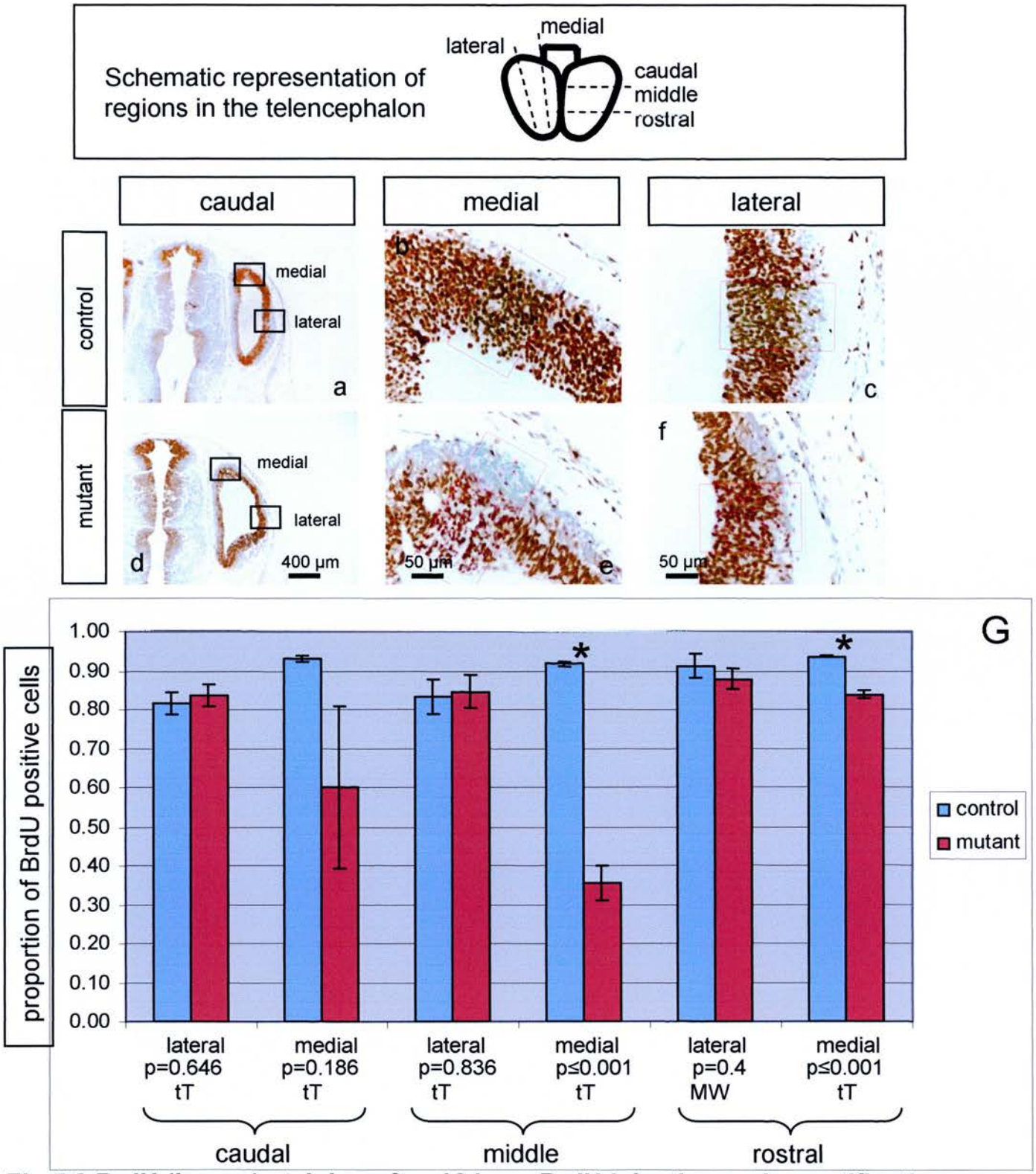


Fig 5.3 BrdU (brown) staining after 12 hour BrdU injection and quantification of BrdU-positive cells in the DTel of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5 Immunostaining shows that both the control (a, b, c) and the mutant (d, e, f) incorporate BrdU.. Quantification of BrdU positive cell number expressed a proportion of all cells (g) shows that BrdU expression is significantly decreased in the mutant DTel in middle-medial region. tT-t-Test performed. MW-Mann-Whitney test performed. N=3 embryos. Error bars are SEM. *-significant difference. MW test is used when normality of equal variance test failed. Blue staining – haematoxylin

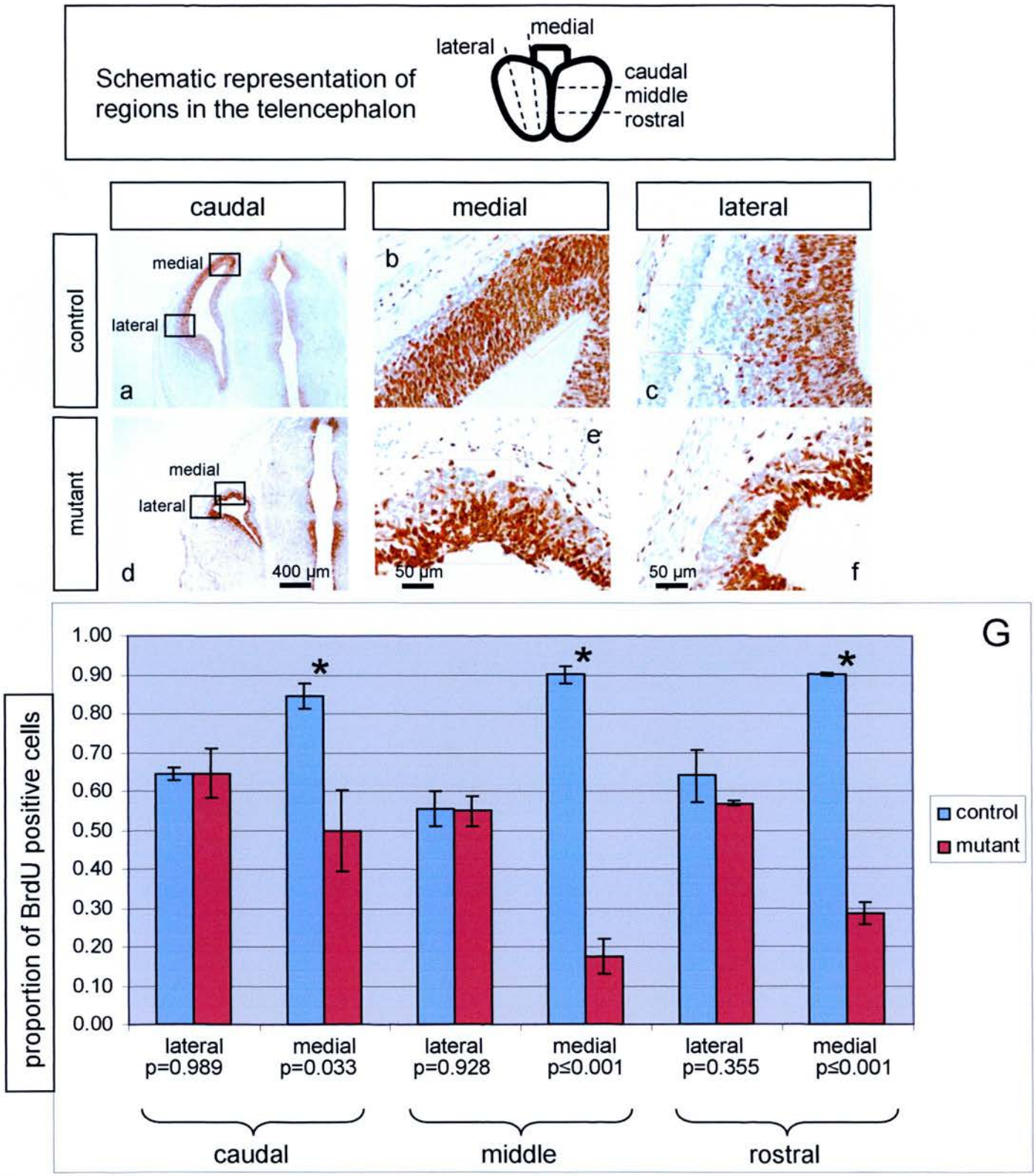


Fig 5.4 BrdU (brown) staining after 12 hour BrdU injection and quantification of BrdU-positive cells in the DTel of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 Immunostaining shows that both the control (a, b, c) and the mutant (d, e, f) incorporate BrdU.. Quantification of BrdU positive cell number expressed as proportion of all cells (g) shows that BrdU expression is significantly decreased in the mutant DTel in caudal-medial, middle-medial, and rostral-medial region. T-test performed. N=3 embryos. Error bars are SEM. *-significant difference. Blue staining – haematoxylin

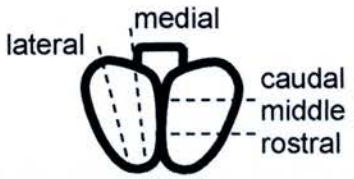
5.2.1 Cell cycle parameters

Although the number of proliferating cells is clearly decreased in the mutant DTel, these findings do not reveal changes in cell cycle parameters. Therefore an analysis of the length of the cell cycle and S-phase was performed to disclose interesting aspects of cell proliferation in the $Emx1^{Cre/+}APC^{580S/580S}$ embryos. Investigation of these parameters was carried out using two nucleotide analogues BrdU and IddU as described by Martynoga et al. (2005). This technique allows us to determine both Tc (cell cycle length) and Ts (S-phase length) from a single experiment. Immunostaining shows that both the control and the mutant incorporate BrdU and IddU both at E12.5 (Fig. 5.5 c-h) and E13.5 (Fig. 5.6 c-h). Quantification of S-phase length did not reveal significant differences between the control and the mutant in any area of the DTel at E12.5 (Fig. 5.5 a). There is no significant difference in S-phase at E13.5 either (Fig. 5.6 a). Cell cycle length quantification at E12.5 shows that in spite of the decrease in the proliferating pool, the cycling cells of the mutant DTel have similar Tc compare to the control (Fig. 5.5 b). Analysis of cell cycle duration at E13.5 did not reveal significant differences between the mutant and control cycling cells in the DTels (Fig. 5.6 b), although the mutant has a decreased proliferative pool in the DTel.

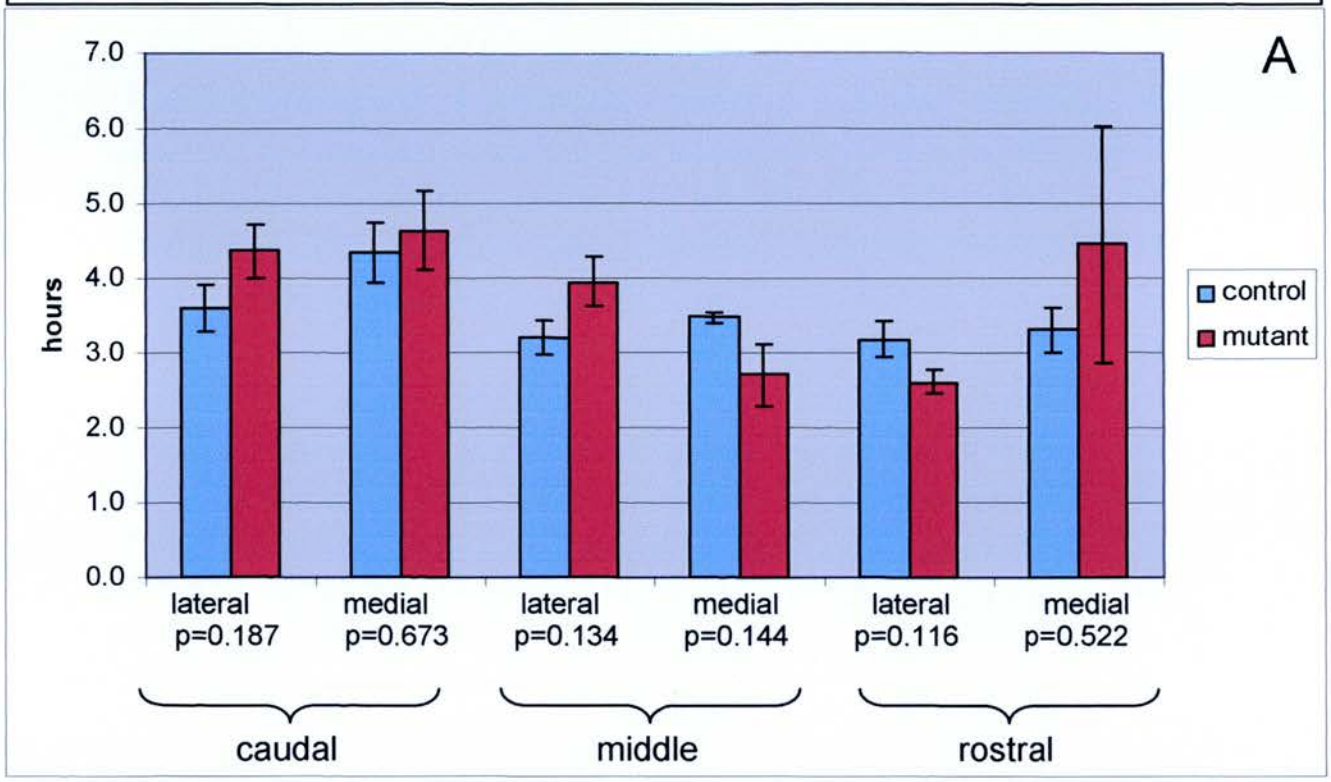
5.2.2 Cell cycle phases

Further investigation of proliferation was done by using FACS analysis, which allows the designation of cells to G0/G1, S, or G2/M populations according to their content of DNA. Cell sorting was performed at E13.5 on the DTel and ventral

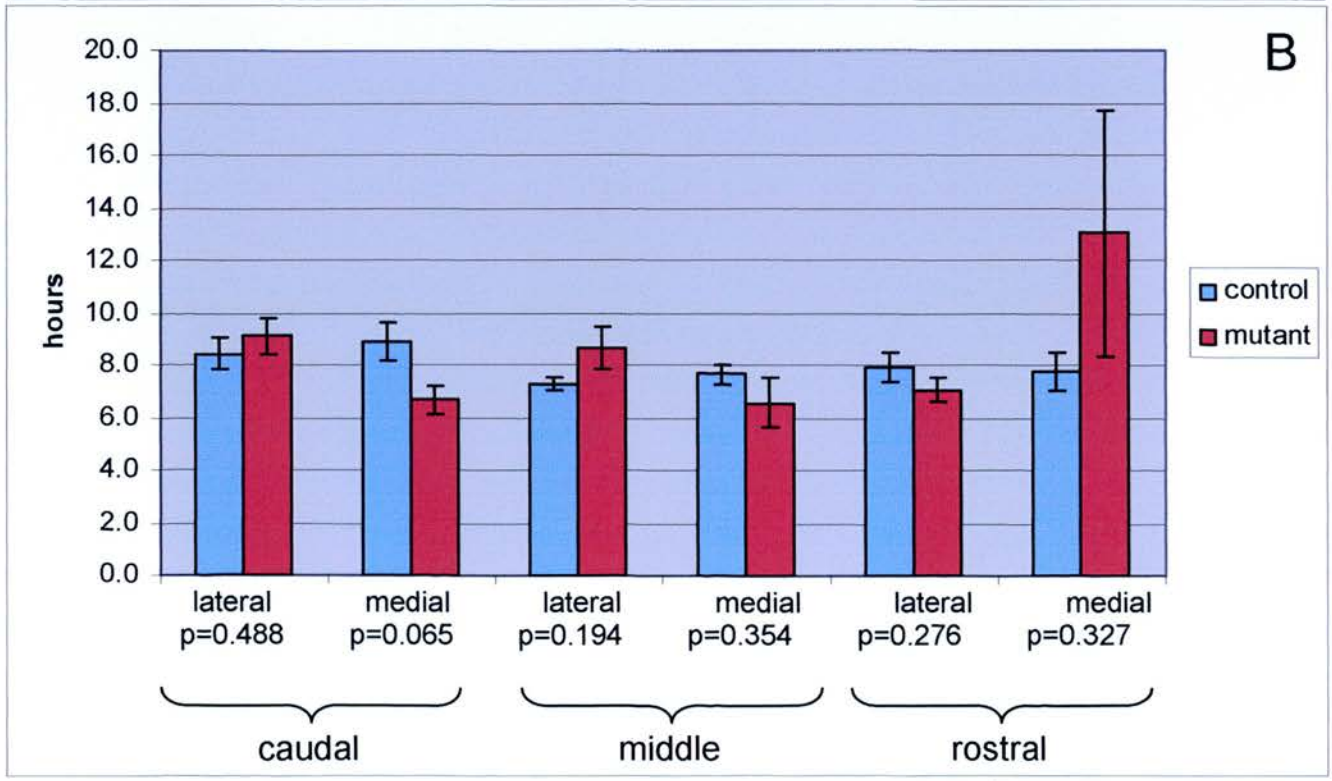
Schematic representation of regions in the telencephalon



E12.5 S-phase length



E12.5 cell cycle length



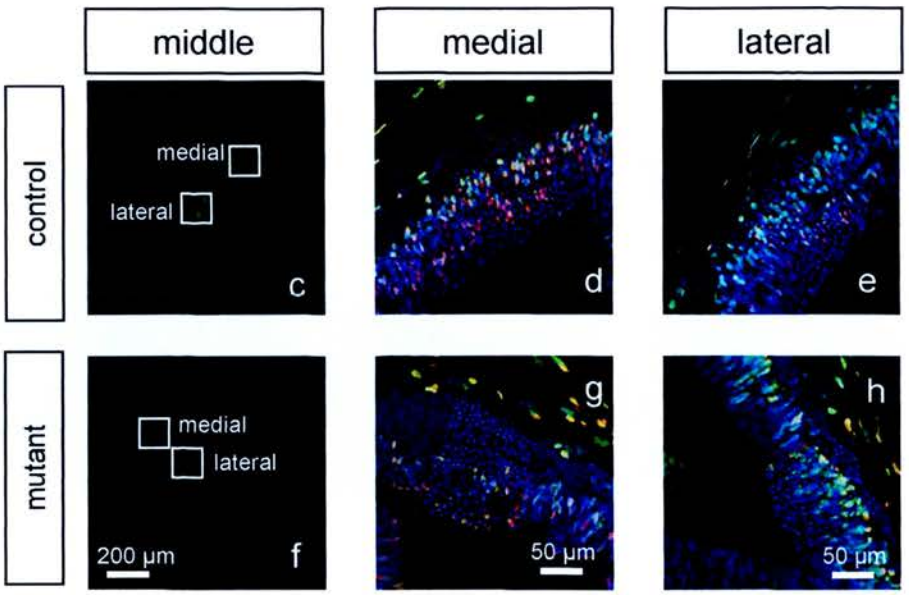
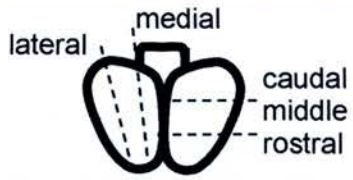
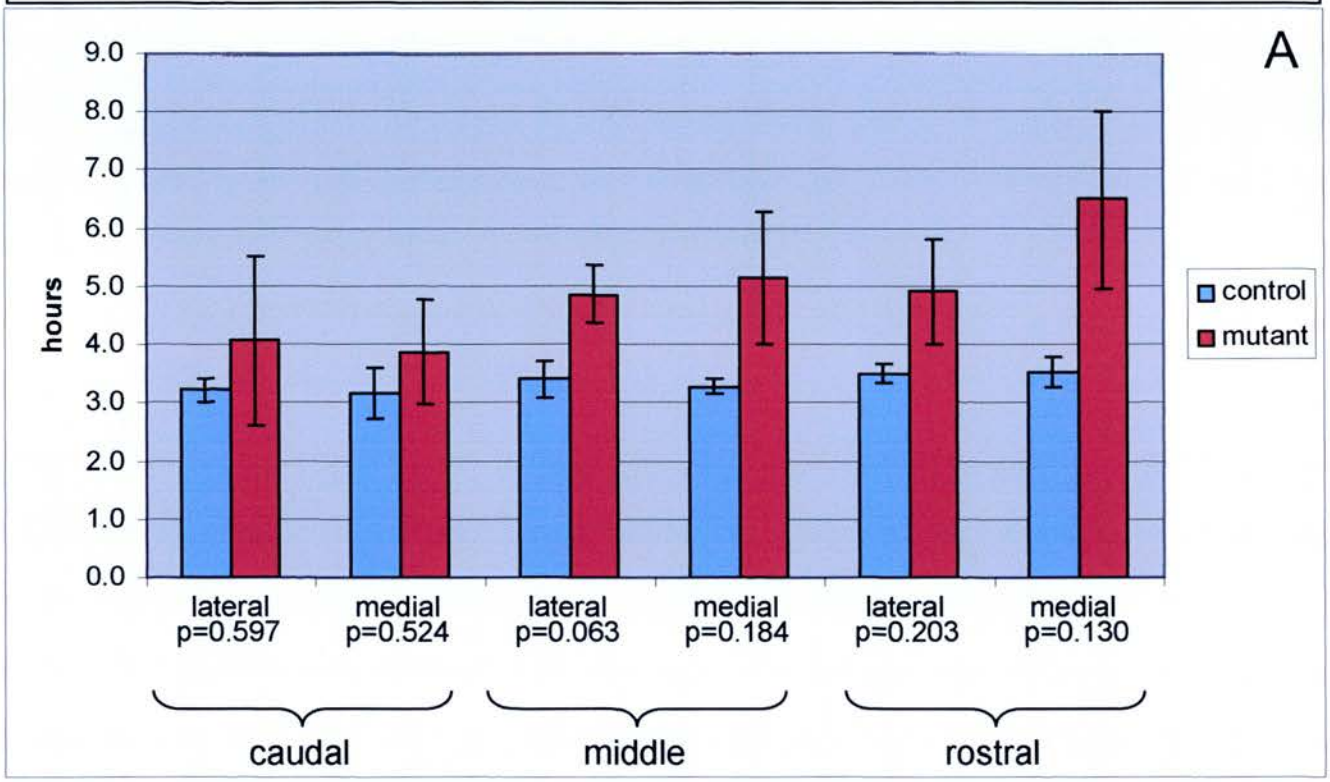


Fig 5.5 S-phase and cell-cycle length quantification after IddU/BrdU pulse of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E12.5 Quantification of S-phase length (a) shows that there is no significant difference between the control and mutant in any region. Quantification of cell cycle length (b) shows that there is no significant difference between the control and mutant in any region of the DTel. Immunostaining shows that both the control (c, d, e) and the mutant (f, g, h) incorporate BrdU and IddU. T-test performed. N=3 embryos. Error bars are SEM. *-significant difference. White dots indicate nuclei, pink dots indicate IddU/BrdU (red) positive cells, yellow dots indicate BrdU (green) only positive cells. Blue staining – ToPro-3

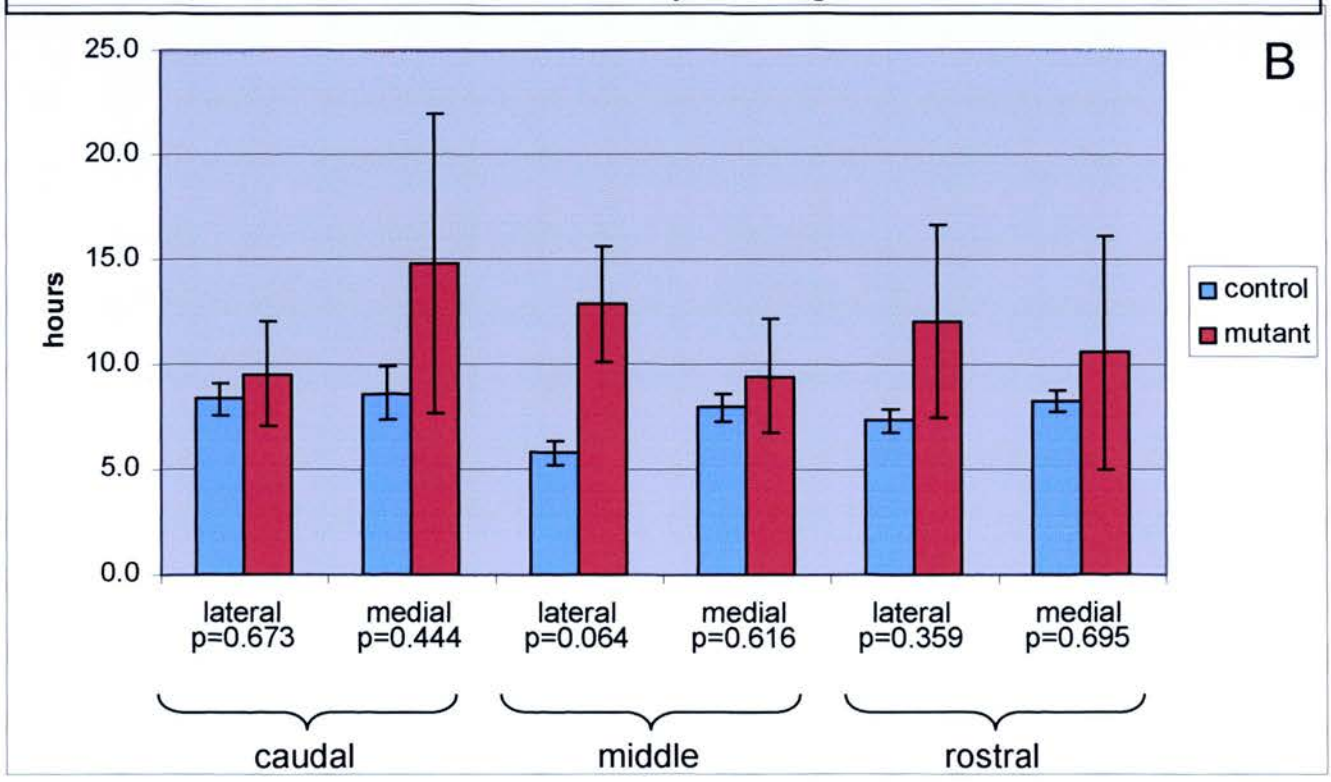
Schematic representation of regions in the telencephalon



E13.5 S-phase length



E13.5 cell cycle length



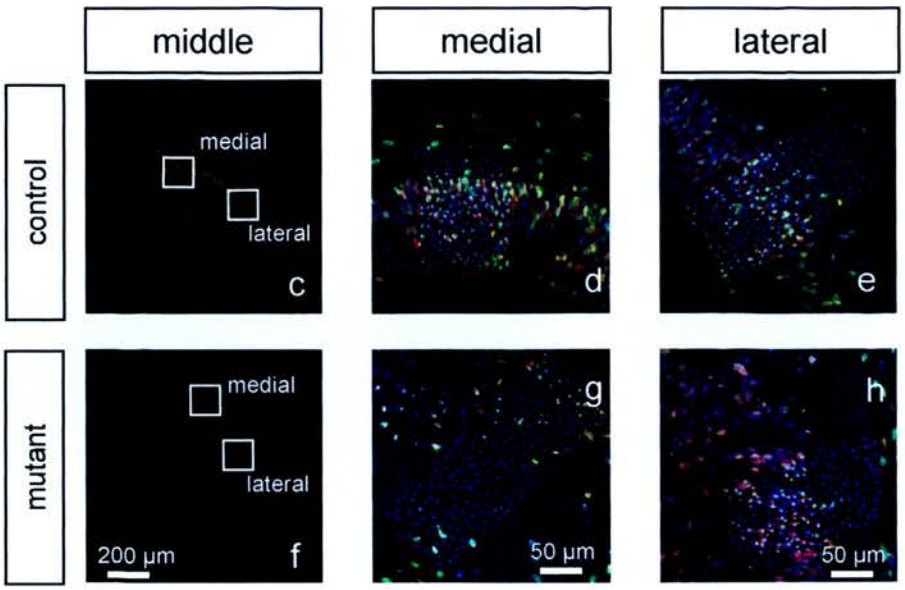


Fig 5.6 S-phase and cell-cycle length quantification after IddU/BrdU pulse of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 Quantification of S-phase length (a) shows that there is no significant difference between the control and mutant in any region of the DTel. Quantification of cell cycle length (b) shows that there is no significant difference between the control and mutant in any region of the DTel. Immunostaining shows that both the control (c, d, e) and the mutant (f, g, h) incorporate BrdU and IddU. T-test performed. N=3 embryos. Error bars are SEM. *-significant difference. White dots indicate nuclei, yellow dots indicate IddU/BrdU (red) positive cells, light blue dots indicate BrdU (green) only positive cells. Blue staining – ToPro-3

telencephalon of the control and the mutant using Propidium Iodide as a DNA label. Results obtained from the DTel show an almost halving of the S and G2/M populations in the mutant compared to the control (Fig. 5.7 tables of b and a, respectively). In agreement with this is an increase of the G0/G1 population in the mutant compared to the control (Fig. 5.7 tables of b and a, respectively). The ventral telencephalons were used to show that this region is unaffected in the mutant. FACS analysis of cells of the ventral telencephalon did not reveal any obvious differences in distribution of cells to a particular population in the control and mutant (Fig. 5.8 tables of a and b, respectively).

Thus, $Emx1^{Cre}$ - mediated deletion of *Apc* in the DTel leads to a decreased proliferative pool in the affected areas, and those cells which are able to cycle do so at similar rate to those found in the control embryos. In the ventral telencephalon, where *Apc* is not deleted, cell distribution to particular phases is unaffected in the mutant.

5.3 Aneuploidy

As was shown by a number of groups, mutation of *Apc* can cause chromosome number abnormalities (Fodde et al., 2001; Tighe et al., 2004; Dikovskaya et al., 2007). FACS analysis was used to look for evidence of abnormality of chromosome number. The limit of resolution of this technique is about 5% of intracellular DNA content (Crompton et al., 2002). However an obvious limitation is the impossibility of detecting

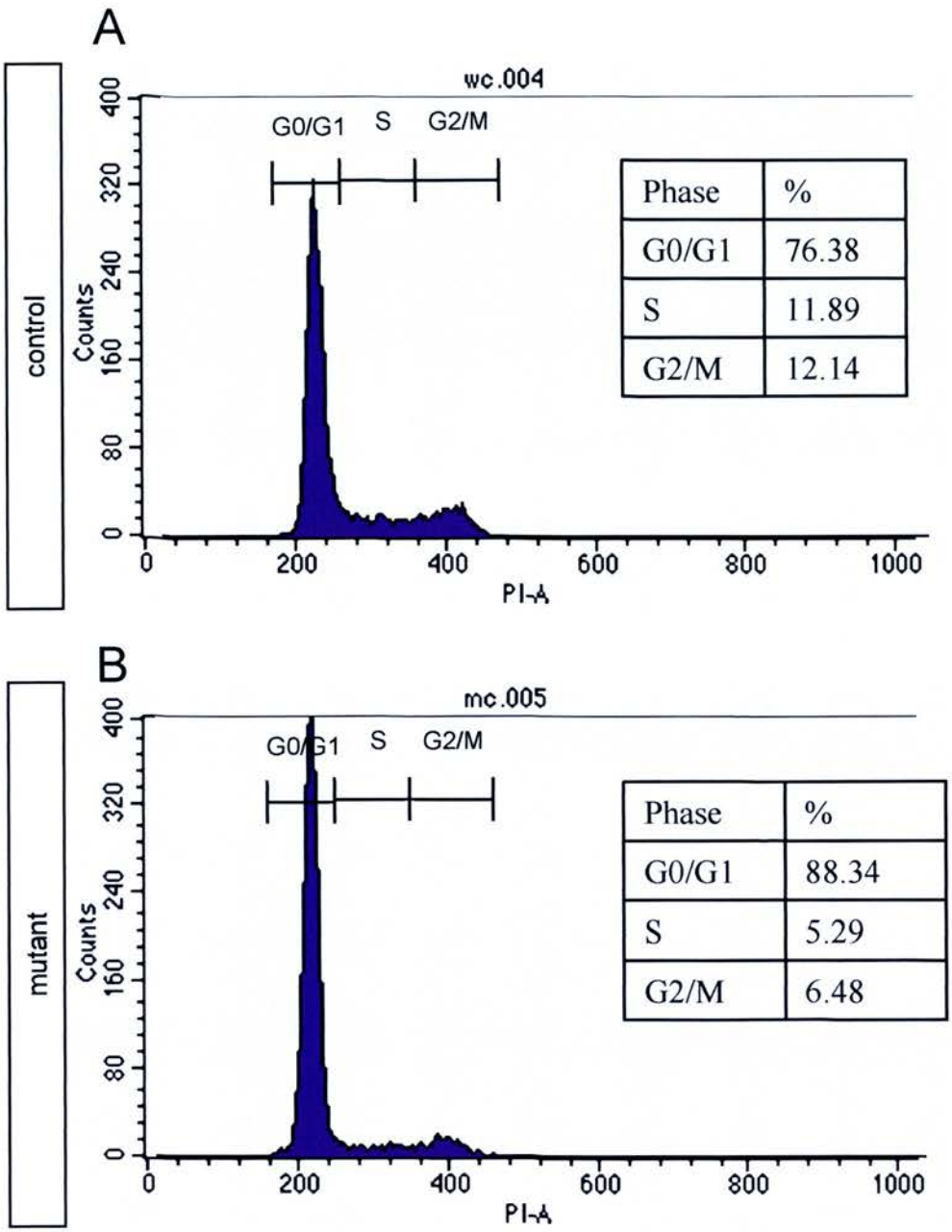


Fig 5.7 FACS analysis of DNA content using Propidium Iodide in DTel cells of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 There are more cells in G0/G1-phase in the mutant (b, table) compare to the control (a table). S and G2/M number is decreased in the mutant (b, table) compared to the control (a, table). Assignment of cell cycle phases was done using CellQuest software for FACS. There are no signs of ploidy defects as both graphs do not have peaks outside of 2n-4n areas (a and b). N=2 embryos

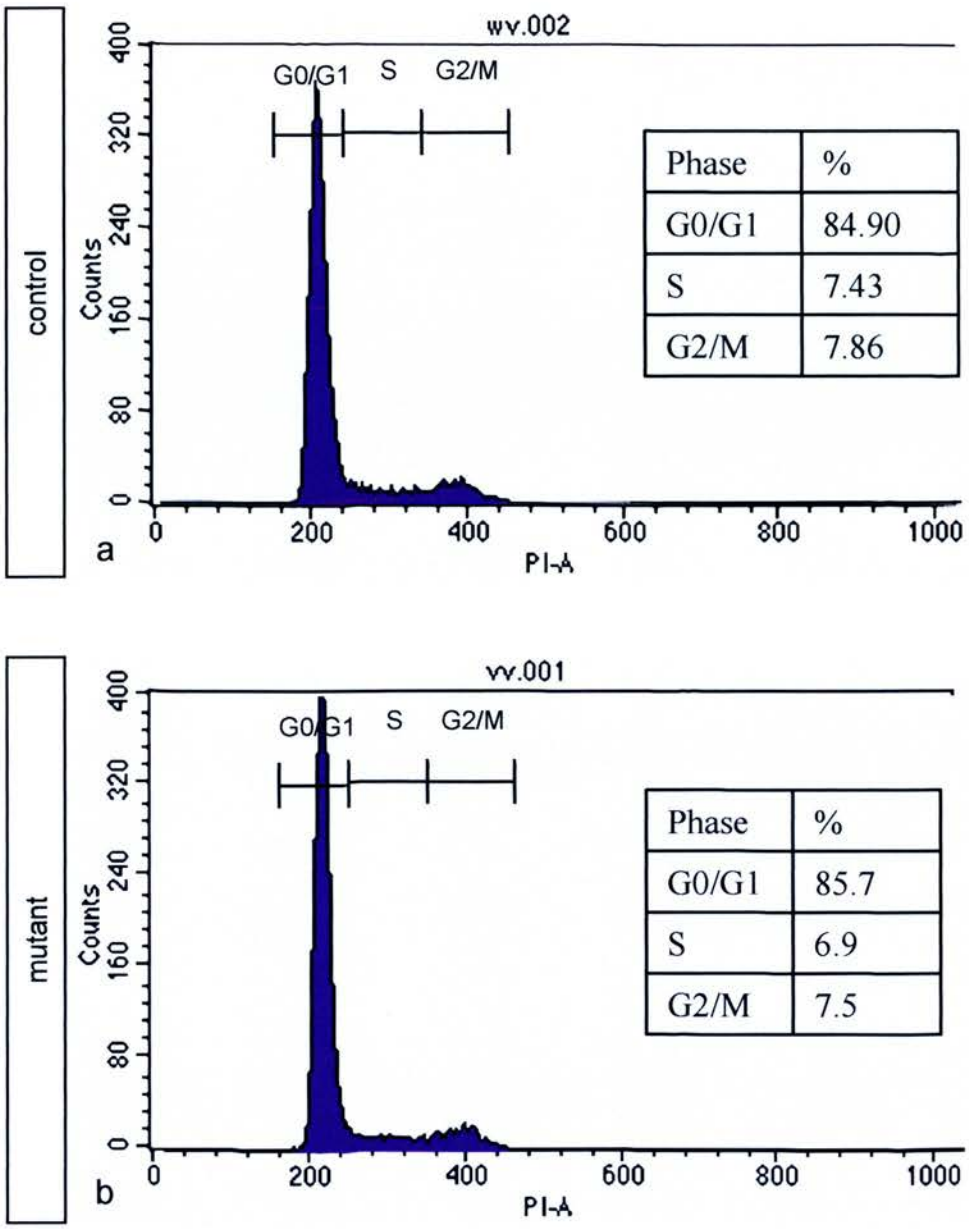


Fig 5.8 FACS analysis of DNA content using Propidium Iodide in the ventral telencephalon cells of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5 There is no difference in content of different cell population in the control (a, table) and the mutant (b ,table). Assignment of cell cycle phases was done using CellQuest software for FACS. There are no signs of ploidy defects as both graphs do not have peaks outside of 2n-4n areas (a and b). N= 2 embryos

chromosome translocations, but translocations are not common in *Apc* mutant cells (Prall et al., 2007)

A graph presenting the DNA content of cells of the DTel from the control ($Emx1^{Cre/+}APC^{580S/+}$) embryo (Fig 5.7 a) shows a characteristic shape. There are no additional peaks in the area of the plot that corresponds to less than 2n DNA content (Fig 5.7 a, graph). Also there is no sign of polyploidy, as there is no signal in areas corresponding to more than 4n (Fig 5.7 a, graph). The DNA content profile of mutant DTel cells is similar to the control profile (Fig 5.7 b, graph). Also there are no additional peaks outside 2n-4n area (Fig 5.7 a, graph).

According to these data, the presented Apc mutation at E13.5 does not have detectable signs of ploidy abnormalities, namely aneuploidy and polyploidy.

5.4 Apoptosis

Regulation of apoptosis is crucial for proper embryonic development of various structures (reviewed by Pettmann and Henderson, 1998). P53 protein was described as a central molecule in cell death regulation, which stimulates apoptosis (Gottlieb and Oren, 1998). However apoptosis can be promoted in other (p53-independent) ways (Degenhardt et al., 2002). Apc is referred to as tumour suppressor protein (Smith et al., 1993). The tumour suppressor activity of Apc is thought to be mostly as a reflection of

its ability to regulate beta-catenin levels (Munemitsu et al., 1995). As was mentioned before, there are pro-apoptotic or anti- apoptotic activities of *Apc* mutations (Morin et al., 1996; Hasegava et al., 2002; Venesio et al., 2003), although, Cre-loxP experiments using the same *580S* allele that we have used in this study showed increased apoptosis when *Apc* was mutated (Sansom et al., 2004; Hasegava et al., 2002). Therefore it was of interest to determine levels of apoptosis in the DTel of $Emx1^{Cre/+}Apc^{580S/580S}$ embryos.

Investigation of apoptosis by TUNEL staining revealed an increased number of apoptotic cells in the DTel of the mutant compared to the control at E13.5 (Fig. 5.9 d, white arrows, compare to b, respectively). Quantification performed in three areas of the DTel (rostral, middle and caudal) found that there is a significant increase in cell death in all three regions in the mutant compared to corresponding regions of the control DTel (Fig 5.9 e).

Quantitative RT-PCR analysis of *p53* expression was performed at three different stages: E12.5, E13.5, and E15.5. We found no significant up-regulation of *p53* expression at any of the stages examined (Fig 5.10).

In conclusion, apoptosis is increased in the mutant DTel in all regions, but its mechanism seems to be p53 independent.

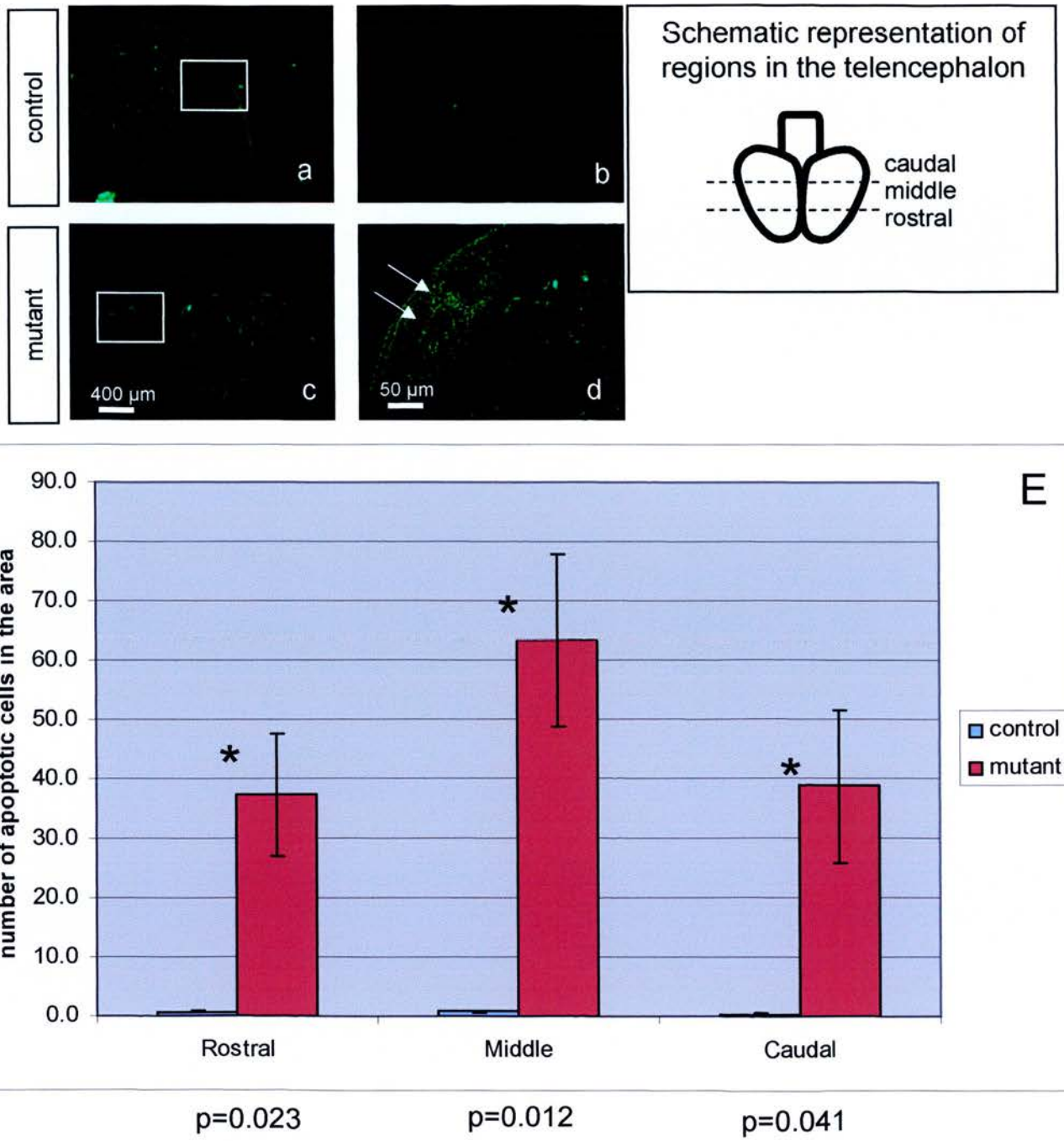
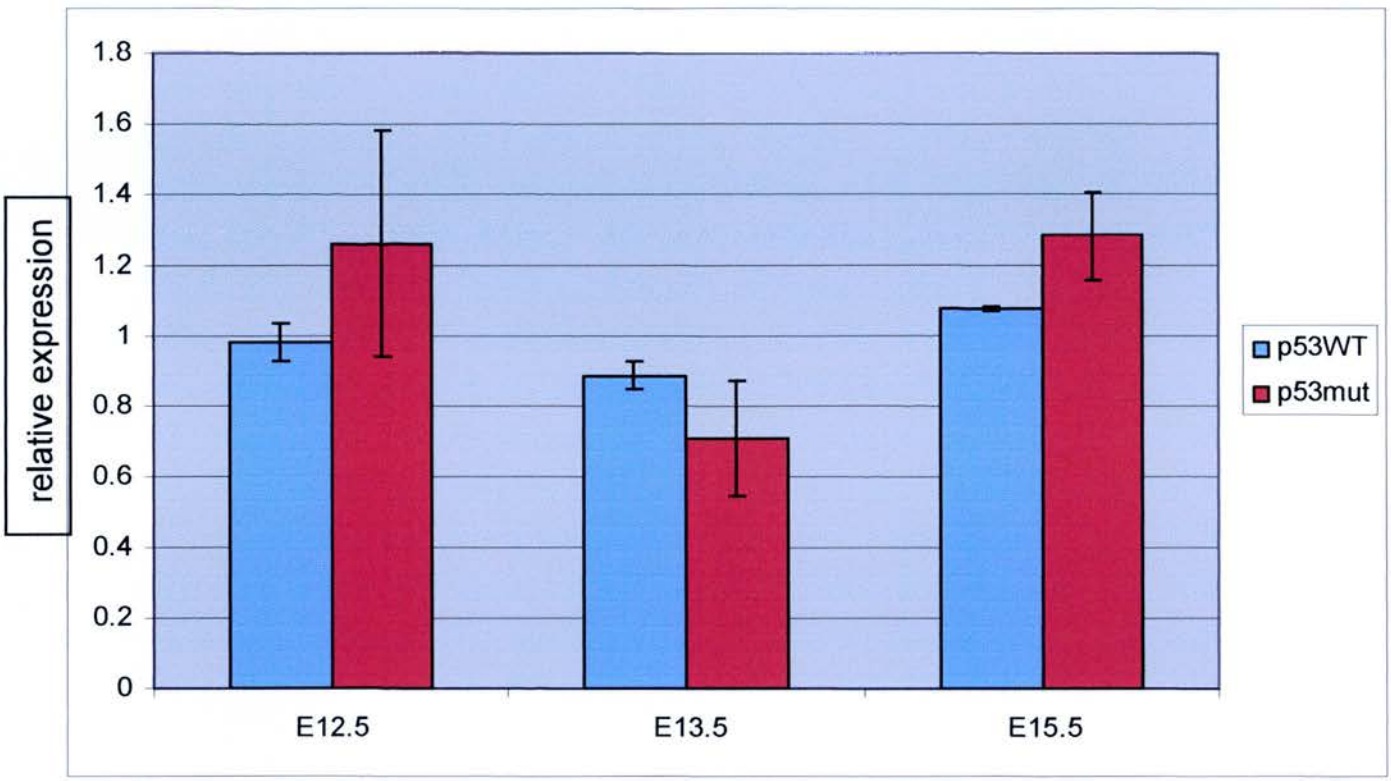


Fig 5.9 TUNEL (green) staining and DTel apoptotic cell quantification of the control (*Emx1*$^{Cre/+}$*Apc*$^{580S/+}$) and mutant (*Emx1*$^{Cre/+}$*Apc*$^{580S/580S}$) embryos at E13.5
The mutant shows more apoptotic cells in the middle of the DTel (d, white arrow) compare to the control (b). Apoptosis quantification shows that there is significantly more apoptosis in the mutant compared to the control (e) in all areas: rostral, middle, and caudal T-test performed. N=3 embryos. Error bars are SEM. *-significant difference.



P =0.250 (MW) P =0.346 (tT) P =0.187 (tT)

Fig 5.10 Quantitative RT-PCR of *p53* expression in the telencephalon of in control (*Emx1Cre/+Apc580S/+*) and mutant (*Emx1Cre/+Apc580S/580S*) embryos at E12.5, E13.5 and E15.5. Expression levels of *p53* in the control and the mutant are similar at any age. MW -Mann-Whitney test performed, tT - t-test performed. N =3 embryos. Error bars are SEM. *-significant difference. MW test is used when normality of equal variance test failed.

5.5 Discussion

5.5.1 Proliferation

Our original hypothesis was that $Emx1^{Cre/+}APC^{580S/580S}$ embryos would have increased proliferation in the affected region. This assumption was based on the work of Chenn and Walsh (2002), who showed an expansion of the proliferative pool in transgenic embryos expressing stabilized beta-catenin in neural precursors under the control of the *Nestin* promoter. My model utilizes *Emx1,* which is expressed in the cortex from E9.5, when it consists of the proliferating neuroepithelium layer (Gulisano et al., 1996). Expression of *Emx1* can be found in proliferating, differentiating, or migrating precursors and cortical neurons throughout development of the DTel (Gulisano et al., 1996). Thus, there is evidence that beta-catenin is stabilized in proliferating cells due to *Apc* mutation. It was shown in chapter 3 that beta-catenin is stabilized in the mutant DTel from E10.5, while neurogenesis in the DTel starts at around E11.5 with maximum neurogenesis around E12.5-13.5 (Soriano et al., 1995). Additional support of the hypothesis that proliferation may be increased in $Emx1^{Cre/+}APC^{580S/580S}$ embryos can be found in the work of Chenn and Walsh (2002) who showed that activation of canonical Wnt signaling drives proliferation after beta-catenin stabilization.

Results from my mutant embryos at E12.5 and E13.5 presented in this chapter show that the mutant DTel has a decreased proliferating pool, and that cells cycle at about the normal rate.

A possible explanation is suggested by PCNA and BrdU analysis of the proliferative pool in the mutant DTel. Continuous BrdU labeling for 12 hours reveals a more dramatic decrease in the proliferative pool than is suggested by PCNA labeling. This suggests that some cells expressing PCNA are not actually cycling. It is important to notice that the PCNA level in the nucleus changes during the cell cycle: it is highest in S-phase (Beppu et al., 1994). One of PCNA's binding partners is p21 (Koundrioukoff et al., 2000). It is known that p21 controls the G1/S transition by binding PCNA and thus suppressing its functions in DNA replication (Shteiman et al., 1994). Immunostaining for p21 showed a strong increase of its expression in the dorsal telencephalon (chapter 3). Therefore it is possible that up-regulation of p21 may lead to binding of PCNA and cell cycle arrest in G1 phase. FACS data show good agreement with G1/S transition blockade as the population of G0/G1 cells is increased and S and G2/M populations are decreased (Fig 5.7).

Also it is possible to speculate that after Apc deletion Wnt signaling becomes deregulated and the DTel changes its identity as a result of this (chapter 4). Expression of the telencephalic marker Foxg1 is lost in the mutant DTel at E12.5 (Chapter 4). Hanashima et al. (2002) suggest that the telencephalon of *Foxg1*$^{-/-}$ embryos has increased neuronal production as a result of premature differentiation and decrease in the proliferative pool. This can be consistent with observations done in our mutants: the neuronal marker Tuj1 shows increased expression in the mutant DTel medially at E13.5

(chapter 3) and a decrease in progenitor cell marker Nestin expression (Chapter 3). However the DTel of Foxg1 mutants show almost a doubling in the length of the cell cycle (Martynoga et al., 2005). But cell cycle length in the $Emx1^{Cre/+}Apc^{580S/580S}$ is similar to that of the control. Also at E15.5 another neuronal marker Tbr1 does not show increased neuron production in the mutant DTel. However a possible explanation can be that cells with mutant Apc exit mitosis and begin to express postmitotic neuron markers, but mutant cells subsequently die (discussed below). The decreased number of cells expressing neuronal marker from E15.5 reflects the diminished proliferative pool. Thus, loss of Foxg1 expression in the mutant DTel might explain increased expression of neuronal markers and decrease in expression of progenitor cell markers at E13.5, but the "loss of Foxg1" hypothesis is unable to explain other defects in the mutant.

In order to understand the relationship of p21 to *Apc* mutation there is a question to be answered: how it is induced? Normally the concentration of p21 is low but it can be increased by different factors, such as p53 activation, DNA abnormalities or expression of Wt1 (Chen et al, 1995; Kuwano et al., 1997; Englert et al., 1997). Quantitative RT-PCR did not reveal any increase in the expression of *p53*. It is possible that the p21 up-regulation is p53-independent providing indirect evidence that DNA is not damaged in the mutant (p53 induction in response to DNA damage is shown by Yang et al., 1998). FACS analysis showed no differences in DNA content between the control and the mutant (Fig. 5.7 and Fig. 5.8). The lack of up-regulation of p53 expression in the mutant provides additional though indirect evidence that $Emx1^{Cre/+}APC^{580S/580S}$ cells remain euploid as there are data suggesting up-regulation of

p53 in response to ploidy defects (Steweart et al., 2001). There is a different situation with Wt1 expression. Wt1 is up-regulated in cells positive for nuclear beta-catenin from E12.5 (chapter 4). Moreover it was shown that Wt1 can induce p21 independently from p53 (Englert et al., 1997). Other aspects of Wt1 regulation in respect to Wnt signalling are discussed in chapter 4.

Thus one possible chain of events leading to a halt in G1/S transition and subsequent decrease of the proliferative pool is as follows (Fig 5.11): Apc mutation causes beta-catenin stabilization, which mimics canonical Wnt signalling activation that in turn promotes expression of Wt1; the following step is stimulation of p21 expression, which binds PCNA and causes cell cycle arrest in G1-phase.

5.5.2 Apoptosis

The observed increase in apoptosis in the mutants could be due to several different possible mechanisms. In the majority of cases apoptosis is induced by p53, which mediates the cellular response to various stimuli (Caelles et al., 1994). Jaiswal and Narayan (2001) provided data suggesting p53-dependent cell-cycle arrest and apoptosis of colon cancer cells. However as described in the previous section, I found no evidence of *p53* up-regulation in the Apc^{580S} mutant. As was described before, cell cycle progression control is a function of p21 (Steiman et al., 1994). However its role in apoptosis is controversial. Cancer studies show that p21 promotes cell cycle arrest after p53 stimulation but apoptosis was induced after cleavage of p21 by caspases (Zhang et al., 1999). However other researchers have shown that p21 promotes apoptosis in

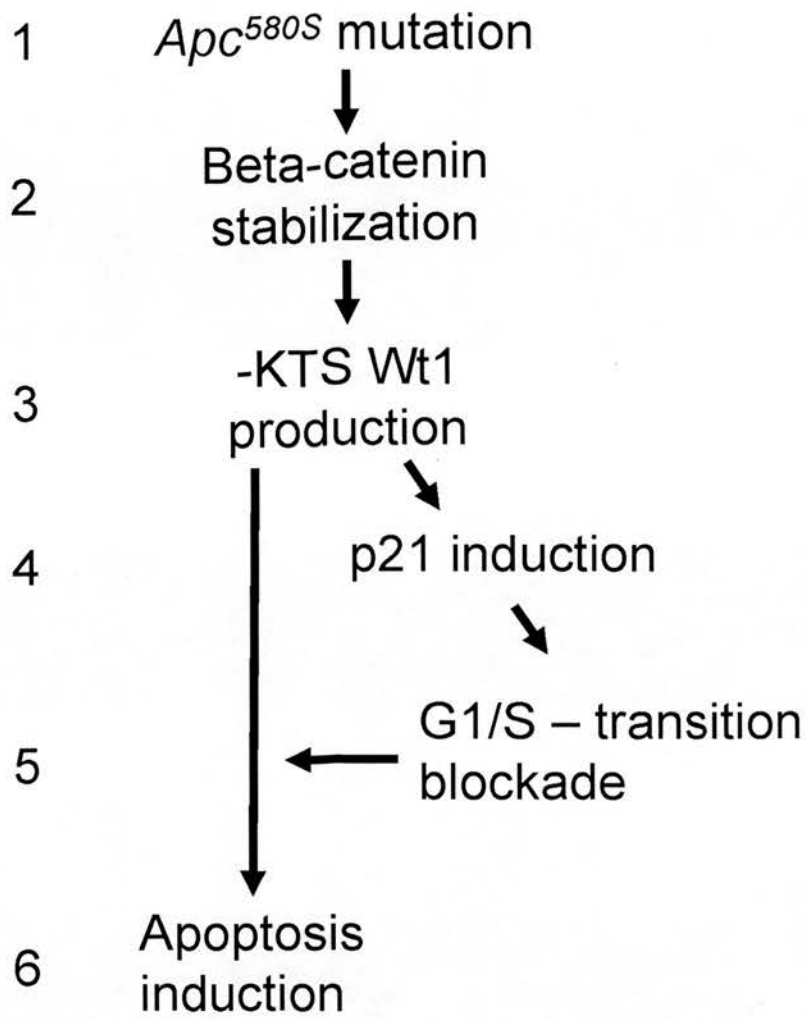


Fig 5.11 Schematic representation illustrating a hypothesis of apoptosis induction. *Apc*^{580S} mutation (1) leads to beta-catenin stabilization(2). (2) induces production of -KTS isoform of Wt1(3), which in turn induces p21(4). This causes the G1/S-transition arrest(5). –KTS Wt1(3) induces apoptosis (6) when the G1/S transition(5) is blocked.

malignant glioma cells (Kondo et al,. 1996). The connection between p21 expression and Wt1 levels suggests a possible explanation of the increased apoptosis level seen in $Emx1^{Cre/+}APC^{580S/580S}$ embryos. It was shown that osteosarcoma cells undergo apoptosis if Wt1 is induced (Englert et al., 1997). However stimulation of apoptosis happens if there is prior G1 arrest associated with p21 up-regulation (Englert et al., 1997). Induction of apoptosis described in this study was related to suppression of epidermal growth factor receptor (EGFR) expression by Wt1. Whether Wt1 can mediate similar mechanism to induce apoptosis in the DTel is unknown. Moreover only the –KTS Wt1 splice isoform (which has additional DNA binding properties) was able to promote cell death and induce p21 expression (Englert et al., 1997). As was shown by Englert with co-workers (1995) Wt1 splice isoform has different localisation in the cells: –KTS spice isoform is more prone to localization in the nucleus than others. Therefore changes in the Wt1 staining pattern from cell membrane only in the control embryos to the nuclear in the mutant embryos might reflect a change in isoform production, providing a suggestion of Wt1 involvement in apoptosis in the mutant. Also Wt1-stimulated apoptosis could explain the decrease in postmitotic neuron marker positive cells observed between E13.5 to E15.5 as described in the previous section. If we accept an idea that a decrease in neurons is related to reduction of Wt1 positive cells at same ages (Chapter 4), than it is possible to assume that these cells die out as they have increased apoptosis due to Wt1 up-regulation.

Thus, a possible sequence of steps leading to increased apoptosis could be the following: -KTS Wt1 isoform production is induced by activated Wnt signalling; then

Wt1 stimulate expression of p21, which causes G1 arrest; the next step is induction of apoptosis (Fig 5.11), but the precise mechanism of apoptosis induction is not known.

5.6 Conclusion

In conclusion, my data suggest that the observed proliferation and apoptosis defects in $Emx1^{Cre/+}APC^{580S/580S}$ embryos may have a common element in their regulation. They have a connection through p21 expression, which in the first case reflects the decrease in the size of the proliferative pool and in the second case is related to apoptosis induction. P21 induction might be associated with over-activation of the canonical Wnt signaling and following Wt1 up-regulation. Thus, the Apc^{508S} mutation exhibits a decrease in proliferative activity possibly due to cell cycle arrest or early mitosis exit. The relation between these mechanisms is obscure. Apoptosis increase is p53 independent and promoted by Wt1 up-regulation through an obscure pathway.

6. Summary and future perspectives

The central nervous system expresses *Apc* at high level during embryonic development (Bhat et al., 1994). Apc is a multifunctional protein, which is involved in many signaling pathways that are essential for development. Our current knowledge of the Apc functions in central nervous system development is not complete.

$Emx1^{Cre/+}Apc^{580S/580S}$ embryos provided a preliminary insight into the roles of Apc in the developing dorsal telencephalon. Usage of a conditional knock-out model was dictated by the fact that $Apc^{-/-}$ embryos die at gastrulation. This problem was solved by using an $Emx1^{Cre}$ conditional knock-out approach to mutate *Apc* specifically in the developing cerebral cortex. As *Emx1* starts to express at E9.5 then the consequences of the mutation are expected after this stage.

My data show that *Apc* is mutated in regions which correspond to regions of *Emx1* expression. Also the medial–lateral *Emx1* expression gradient produces a similar gradient in *Apc* deletion in the dorsal telencephalon: medial regions of the mutant dorsal telencephalon are more affected than lateral. First indirect signs of Apc loss, which are observed at E10.5, are single cells in the dorsal telencephalon with stabilized nuclear located beta-catenin. This observation shows that Apc is important to control beta-catenin translocation to the nucleus and that one day after the deletion is enough to produce the defect. Morphological defects could be found from E12.5: the mutant dorsal telencephalon loses its smooth layer structure. At this age immunostaining reveals loss

of Apc in the cells of the dorsal telencephalon. Quantitative RT-PCR showed *Apc* exon 14 deletion in the mutant. Defects progress with age.

Accumulation of nuclear beta-catenin is a sign of activation of Wnt signalling. Nuclear beta-catenin is able to stimulate the canonical Wnt pathway as shown by BatGal reporter mice and up-regulation of Wnt target genes (C-myc, Cyclin D1). Expression of the negative regulator of canonical Wnt signaling *Axin2* is highly up-regulated which could be an indirect sign of canonical Wnt signaling activation. Cell polarity defects are obvious from E12.5, which suggests a direct effect of Apc loss on the apical-basal cell polarity. Additional evidences of the defect in cytoskeleton regulation are adhesion defect and defect in neuronal processes elongation. Thus, the deletion of *Apc* leads to over-activation of the canonical Wnt pathway and disregulation of cytoskeleton.

Conditional *Apc* mutant embryos exhibit alterations in expression of telencephalic markers in the dorsal telencephalon. Expression of Foxg1 suggests that the mutant dorsal telencephalon changes expression of telencephalic markers from E12.5. There is an expansion of markers of more caudal regions of the brain to the mutant dorsal telencephalon (Pax3, Wnt1). Markers normally expressed in the cerebral cortex (Pax6, Tbr1) are still present but down-regulated. Apical progenitor population and intermediate progenitor pool are decreased. My data show that Apc is important for proper patterning of the cortex probably mostly by antagonizing the canonical Wnt pathway. However a precise mechanism is not yet known.

Involvement of Apc and Wnt signaling in regulation of proliferation suggests an effect on this process. Determination of cell-cycle parameters revealed that S-phase and cell cycle length in 580S *Apc* mutants had no significant difference to those of the

control. Investigation of the proliferative pool by PCNA expression or 12 hour continuous BrdU pulse found that numbers of proliferating cells were decreased. We also found that most cells of the mutant dorsal telencephalon are blocked in the G1 phase. Numbers of blocked cells are increased with the age. This shows that Apc is important for maintenance of the size of the proliferative pool but does not affect the cell-cycle parameters.

The G1/S transition block is possibly related to p21 up-regulation that suggests severe impairment in cellular machinery, which causes cell cycle arrest and apoptosis. It was found that apoptosis is increased in the mutant but level of p53 is not changed, which might reflect activation of p53-independent apoptosis. Apoptosis and p21 expression could be stimulated by Wt1, increased expression of which is observed in the mutant dorsal telencephalon. Up-regulation of *Wt1* expression might reflect its function as a tumour suppressor, which is related to apoptosis induction. In order to stimulate apoptosis Wt1 has to induce cell cycle arrest though up-regulation of p21 expression. These data suggests that removal of *Apc* leads to G1/S cell-cycle arrest with induction of apoptosis through p53-independent pathway.

A known role of Apc in chromosome segregation suggests that *Apc* mutation would cause ploidy abnormalities. But DNA content in the cells was not affected as was confirmed by FACS experiments. Further investigations are needed to confirm these findings.

Thus, the conditional *Apc* deletion demonstrates multiple effects on dorsal telencephalic development. Defects found in the developing dorsal telencephalon include over-activation of the canonical Wnt signaling, defects in cytoskeleton

organization, lost of cell polarity, defects in adhesion, identity defects, and promotion of apoptosis. However there is no complete picture of assignment of these defects to particular functional domains of Apc.

Many questions remained unanswered, which can be investigated in future work. In the current work it was not investigated how removal of *Apc* in the dorsal telencephalon interferes with functions of other regions of the forebrain connected to the dorsal telencephalon (ganglionic eminences, thalamus). Of especial interest is the development of thalamo-cortical and cortico-thalamic projections as cortex loses its identity before generation of projections.

Different populations of cells might react differently to *Apc* mutation. This assumption can be tested by using population specific promoters for *Cre* expression. Also different promoters for *Cre* expression can be used to analyze stage specific importance of Apc.

Another powerful tool for investigation of protein functions is site-specific mutations of different functional domains of *Apc*. This technique would provide a possibility to test importance of different interactions of Apc more specifically during development.

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